

**Chymosin Enzyme Preparation from *Trichoderma reesei*
expressing the Chymosin B gene from *Bos Taurus*
is Generally Recognized As Safe**

Notification Submitted by Genencor, a Danisco Division

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1. GENERAL INTRODUCTION

Chymostar Supreme is the Danisco trade name used for the chymosin enzyme preparation produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding chymosin B from *Bos taurus*. DNA encoding the prochymosin portion of the chymosin protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence and, subsequently, inserted into *T. reesei*.

The enzyme is to be used in the food industry as a processing aid for the manufacture of cheese.

The enzyme is an aspartic endopeptidase (EC 3.4.23.4) which has broad specificity similar to that of pepsin A and clots milk by cleavage of a single 105-Ser-Phe-|-Met-Ala-108 bond in the kappa-chain of casein. It is added to milk to form curds and whey, the first step in cheese manufacture. The curds are then processed further to form cheese and the whey is used as a protein source in many food applications.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor has determined that its chymosin B enzyme preparation from a modified strain of *T. reesei* expressing the chymosin B gene from *Bos Taurus* 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 a chymosin B enzyme preparation produced by a *T. reesei* host, which has been modified to express a gene encoding chymosin B from *Bos taurus*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for food use.^{1,2} The safety of the production organism for this chymosin B, *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. Data showing this chymosin B to be substantially equivalent^{5,7,8-11} to naturally occurring chymosin B is provided in Section 3. 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 Name and Address of Notifier

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1.2 Common or Usual Name of Substance

Chymosin enzyme preparation from *T. reesei* expressing the gene encoding chymosin B enzyme from *Bos taurus*

1.3 Applicable Conditions of Use

The chymosin preparation is intended for use in the cheese making industry for milk coagulation during cheese making. The enzyme preparation is used at minimum levels to achieve the desired effect and according to requirements of normal production following current Good Manufacturing Practices. It is expected that cheese produced using this chymosin will be consumed by the general population.

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 which supports this GRAS determination is enclosed with this letter. 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 of the chymosin B enzyme preparation is *T. reesei* strain GICC03278. It is derived by recombinant DNA methods from strain RL-P37 (see Section 2.2). This genetically modified production microorganism complies with the Organization for Economic Co-operation and Development (“OECD”) criteria for Good Industrial Large Scale Practice (“GILSP”) microorganisms.¹² It also meets the criteria for a safe production microorganism as described by Pariza and Johnson¹ and several expert groups.²⁻⁸ It contains the synthetic chymosin B gene under the regulation of a native *T. reesei* promoter and terminator along with a selectable marker from *A. nidulans* and a *T. reesei* chaperonin gene under the regulation of a different native *T. reesei* promoter and terminator to assist in the excretion of chymosin B from the microorganism.

2.2 Recipient Microorganism

The recipient microorganism used in the construction of the chymosin production strain is a strain of the well-known industrial production strain, *T. reesei*. It is derived from strain RL-P37 obtained from Dr. Montenecourt; the derivation and characterization of strain RL-P37 has been published.¹³ *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina*.^{14,15} Genencor has optimized this strain for the production of enzyme proteins through several gene deletions; the gene deletions did not leave any foreign DNA in the *T. reesei* recipient.

2.3 Chymosin Expression Vector

The transforming DNA consisted of two expression cassettes:

- One expression cassette containing the prochymosin B DNA fused to a *T. reesei* promoter and terminator to control expression and using a well-characterized and recognized selectable marker from *A. nidulans*; and
- The second expression cassette containing a *T. reesei* chaperonin that resides in the endoplasmic reticulum and is involved in folding of nascent secreted proteins fused to a different *T. reesei* promoter and terminator to control expression.

DNA encoding the prochymosin portion of the chymosin B protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence to maximize the gene's expression in the *T. reesei* recipient. 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 presence of the introduced DNA sequences was determined by Southern blot analysis to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism.³⁻⁸ The analysis confirms that the introduced DNA is stably integrated into the *T. reesei* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.¹²

2.5 Antibiotic Resistance Gene

The DNA used for transforming the *T. reesei* host strain does not contain any antibiotic resistance genes.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product. The production organism does not end up in food, and therefore, the first step in the safety assessment as described by the IFBC³ is satisfactorily addressed.

An antibiotic resistance gene was not used in this construction, therefore, there is none present in the enzyme preparation. The production strain does not carry any vectors. As no vector sequences are present in the final strain, the transfer frequency of the integrated expression cassettes is the same as for any other chromosomal sequence. The expression cassettes are completely derived from fungal and synthetic DNA.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Key enzyme and protein chemical characteristics of the chymosin are:

Classification	aspartic endopeptidase
IUB Nomenclature	chymosin
IUB Number:	3.4.23.4
CAS Number:	9001-98-3
EINECS Number:	232-645-0
Reaction catalyzed:	Broad specificity similar to that of pepsin A. Clots milk by cleavage of a single 105-Ser-Phe- -Met-Ala-108 bond in kappa-chain of casein.
Molecular weight:	Approximately 35 kDa
Amino Acid Sequence:	The total nucleotide and amino acid sequences have been determined.

Native chymosin (commercial extracts derived from animals containing the active enzyme rennin) and chymosin preparations (derived from fermentation from other bioengineered microbial strains (*Escherichia coli*, *Kluyveromyces marxianus* var. *lactis*, and *Aspergillus niger* var. *awamori*)) are the subject of GRAS affirmation regulation 21 C.F.R. § 184.1685.¹⁶ The regulation states that the enzyme may be used in food with no limitation other than current good manufacturing practices, and it affirms that this ingredient is GRAS as a direct human food ingredient when used as a processing aid (21 C.F.R. § 170.3(o)(24)) or stabilizer and thickener (21 C.F.R. § 170.3(o)(28)) at levels not to exceed current good manufacturing practice in cheeses (21 C.F.R. § 170.3(n)(5)), frozen dairy desserts and mixes (21 C.F.R. § 170.3(n)(20)), gelatins, puddings, and fillings (21 C.F.R. § 170.3(n)(22)), and milk products (21 C.F.R. § 170.3(n)(31)).

3.2 Amino Acid Sequence

The amino acid sequence of Chymostar Supreme chymosin B is identical to the amino acid sequence of native chymosin B. The amino acid sequence of chymosin B is shown in Appendix I.

3.3 Comparison to other chymosins

Bovine chymosin B is well characterized and is an aspartic protease used as a milk coagulant in cheese-making. It is synthesized in the mucosa of the abomasum (fourth stomach) of unweaned calves as preprochymosin B. Preprochymosin B consists of a 16 amino acid pre-region or secretion signal sequence, a propeptide of 42 amino acids, and the mature chymosin B protein of 323 amino acids. The pre-region directs secretion of the protein and is removed by proteolysis as the protein passes through the membrane from the cytoplasm into the lumen of the endoplasmic reticulum. Prochymosin B is inactive and is secreted to the extracellular space. At low pH (pH 4-5) the propeptide is autocatalytically cleaved to release mature, active chymosin B.

The Genencor chymosin B is identical in sequence to the native chymosin B sequence as specified in the National Center for Biotechnology Information (NCBI) or Swiss-Prot protein sequence databases under accession number P00794. There is a single amino acid difference between the Genencor chymosin B sequence and the sequence of the chymosin B produced by *Aspergillus niger* var. *awamori*, which is the subject of the aforementioned regulation, 21 C.F.R. § 184.1685. This one amino acid difference should not be considered significant as there are different sequences of chymosin B deposited at NCBI, most likely due to different alleles being cloned. These different sequences and their relationship to the P00794 sequence are summarized in the NCBI database entry for P00794.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the chymosin enzyme preparation which follows standard industry practice.¹⁷⁻¹⁹ 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 the chymosin enzyme concentrate are standard ingredients used in the enzyme industry.¹⁷⁻¹⁹ All the raw

materials conform to the specifications of the Food Chemicals Codex, 5th edition, 2003 (“FCC”),²⁰ except for those raw materials which 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. Genencor uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and quality specifications are verified upon receipt. The antifoams used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the production process is <1%.

4.2 Fermentation Process

The chymosin is manufactured by submerged 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 both macroscopically and microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient medium or in an enrichment shake flask followed by a 24-72 hour incubation period.

The fermentation is declared as ‘contaminated’ if infection is observed in 3 successive media plates at a minimum interval of 24 hours.

If a fermentation 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 purification and formulation processes.

4.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment – pH adjustment and cooling
2. Primary separation – rotary vacuum drum filtration
3. Concentration – ultrafiltration and precipitation/centrifugation
4. Dissolution – of precipitate and treatment with cellulose
5. Polish filtration – for removal of residual production strain organisms and as general precaution against microbial degradation

4.3.2 Formulation and Standardization Process

The product is formulated with sodium chloride, propylene glycol, methionine and sodium benzoate and standardized according to the product specification to the right concentration. Caramel color may be added.

4.4 Quality Control of Finished Product

The final chymosin preparation 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 (“JECFA”) in 2006 and the FCC. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative composition

The chymosin enzyme preparation has the following typical composition:

Enzyme: 0.24 -0.30% active protein
Sodium benzoate: 0.26-0.35% (w/w)
Sodium chloride: 10-15% (w/w)
Propylene glycol: 2.5-3.5% (w/w)
Methionine: 0.1-0.15% (w/w)
pH adjustment to 5.8-6.25 with 10% sodium hydroxide
Water: ~80-87%.

5.2 Specifications

The chymosin enzyme preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC. See FCC (2003)²⁰ at 149. In addition, the chymosin enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA.²¹

The following specifications have been established for the chymosin enzyme preparation:

(See next page)

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Chymosin B (International Milk Clotting Units)	IDF 157A	International IDF Standard 157A:1997 – “Determination of total milk-clotting activity”	IMCU/ml 600-750
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - “Microbiology - General guidance for the enumeration of micro-organisms - colony count technique at 30°C” and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - “General guidance for the enumeration of coliforms - colony count technique” and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
<i>E coli</i>	819V	ISO 7251 - Microbiology - “General Guidance for Enumeration of Presumptive <i>Escherichia coli</i> - Most Probable Number Technique” and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g

Property	Method Number	Reference Method	Specification
MICROBIOLOGICAL ANALYSIS (Continued)			
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Production strain	892V	Genencor Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Heavy Metals, as Pb	603W	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests Test solutions, and other reference materials, 1983, 2 nd revision	Less than 30 mg/kg
Arsenic	603W-AS	Same as Heavy Metals as Lead	Less than 3 mg/kg
Cadmium	603W-CD	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Mercury	603W-HG	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Lead	603W-PB	Same as Heavy Metals as Lead	Less than 5 mg/kg
Mycotoxins	604W	Patterson & Roberts, Assoc. of Anal. Chem. (vol. 62, no. 6, 1979)	Negative by test

Heavy metals, lead, arsenic, cadmium, mercury, antibacterial activity, and mycotoxins are analyzed at regular intervals. Activity and microbial specifications are analyzed on every lot.

The lead, Coliforms, and Salmonella specifications meet FCC and JECFA requirements for enzyme preparations cited above. The *E. coli*, antibacterial activity and mycotoxin specifications meet JECFA requirements and are not included in FCC (although FCC mentions mycotoxins, but has not established tolerances). The production microorganism specification is a Genencor specification and is not mentioned in FCC or JECFA.

The chymosin activity assay measures the milk clotting ability of the enzyme in International Milk Clotting Units according to a standard assay from the International Dairy Federation.

6. APPLICATION

6.1 Mode of Action

As noted above, chymosin B causes endohydrolysis of peptide linkages in proteins.

Chymosin B will be used for clotting or coagulation of milk during the manufacture of cheese, just as the other commercial chymosins already on the market are used. The resultant curds will be used to make cheese. The resultant whey can be concentrated to meet various specifications, or it can be separated into the protein and carbohydrate (i.e., lactose) fractions and used as a food ingredient. Whey proteins are often used in nutritional supplements, and low pH beverage products and the lactose is used as a carbohydrate source in many food products.

6.2 Use Levels

In a typical cheese-making process, the recommended dosage for this chymosin B is 50 imcu (approximately 0.06-0.09 ml) of product per liter of milk. This is the same or lower level of use as the use level for chymosin preparations that are the subject of 21 C.F.R. § 184.1685.

6.3 Enzyme Residues in the Final Foods

As with other chymosins, approximately 10% will go into the cheese and 90% into the whey. The 90% in the whey will tend to partition into the protein portion rather than the carbohydrate, or lactose, portion. Depending upon the use of the whey or whey fraction, the chymosin may be inactivated during the food processing steps. The fate of this chymosin is identical to those already in commerce. The residue level is

assumed to be the same as the residue level for the chymosin preparations that are the subject of 21 C.F.R. § 184.1685.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism is the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food.² If the organism is nontoxic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using procedures that adhere to FDA's food CGMPs under 21 C.F.R. Part 110, are safe to consume.³ Pariza and Foster² define a nontoxic 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 nonpathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *T. reesei* meets these criteria for nontoxigenicity and nonpathogenicity.

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 that *T. reesei* be placed into synonymy with *T. longibrachiatum*.²² Later however, there subsequently appeared evidence that the two species were not identical.²³ The proposal by Kuhls et al.¹⁴ 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, *T. reesei*, *T. longibrachiatum*, and *H. jecorina* may appear in different documents, but they refer to essentially the same microorganism species.

A review of the literature search on the organism (1972-2006) uncovered no reports that implicate *T. reesei* in connection with a disease, 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 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.

Bruckner and Graf reported the isolation from *T. reesei* strain QM 9414 (a different strain than the strain producing the chymosin that is the subject of this notification) of a peptaibol compound that exhibited antibiotic activity.²⁴ Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains.²⁵ 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, has been a safe producer of commercial cellulase enzyme preparations for food applications.²⁶ 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.²⁶, Blumenthal²⁷ and Olemska-Beer et al.²⁸ 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 paper¹ and various strains have been approved to produce commercial enzyme products internationally, for example, in Canada²⁹, the United States³⁰, France³¹, Australia/New Zealand³², China (MOH 1996)³³, and Japan³⁴. In addition, JECFA has reviewed cellulase from *T. reesei* and set an ADI of not specified for the enzyme.³⁵

The production organism of the chymosin B enzyme preparation, the subject of this submission, is *T. reesei* strain GICC03278. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to express the bovine chymosin B 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.¹³ 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 (see Appendix 3). One of these strains, modified to overexpress an endoglucanase enzyme, was tested for pathogenicity and toxicogenicity in an acute intraperitoneal study in rats and determined to be nonpathogenic and nontoxicogenic. Another study on strain A83, also derived from RL-P37 through mutation and selection, also determined the strain to be nonpathogenic and nontoxicogenic (see Appendix 3 for a summary of the toxicity data on these *T. reesei* strains and others). As Dr. Pariza states in his June 19, 2007 opinion, “*T. reesei* RL-P37, and enzyme preparations derived from this organism including cellulase, beta-glucanase, xylanase, acid fungal protease, alpha-amylase and glucoamylase, are well recognized as safe by qualified experts. 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 Genencor’s unpublished safety studies, support this conclusion. The new chymosin B production strain, *T. reesei* Pent CHY-Bip3 [also labeled GICC03278] is derived from this safe strain lineage.” (See Appendix 4.)

In accordance with the Pariza opinion, Genencor found there was no need to conduct any additional testing of the GICC03278 strain beyond that which was already performed (see Appendix 5).

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 chymosin B, and the GIGC03278 strain is non-pathogenic and non-toxicogenic.

7.1.1 Safety of the Donor Source

The donor organism for the prochymosin B gene used in construction of the new microorganism *T. reesei* GICC03278 was, indirectly, *Bos taurus*. The amino acid sequence of an allelic form (the B form) of bovine preprochymosin is available in the NCBI or Swiss-Prot protein sequence databases under accession number P00794. DNA encoding the prochymosin portion of the protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence. Therefore, the gene for chymosin and the resultant product, chymosin, are recognized as safe.

7.1.2 Safety of the Strain Lineage

QM6a-derived strains account for nearly all industrially produced (by fermentation) cellulase in the world.²⁴ The derivation and characterization of strain RLP-37 has been published by Sheir-Neiss and Montencourt¹³, which Genencor used to produce cellulases for over fifteen years. Genencor has developed many production strains from it using recombinant DNA techniques. One of these strains, modified to overexpress an endoglucanase enzyme, has been tested for pathogenicity and toxicogenicity in an acute intraperitoneal study in rats and was determined to be nonpathogenic and nontoxicogenic. Another strain A83, also derived from RL-P37 through mutation and selection, was also determined to be nonpathogenic and nontoxicogenic. See Appendix 3 for a summary of the toxicity data. Genencor established that all of the products produced by the QM6a-derived strains are safe for their intended uses. The toxicology testing conducted on the enzymes developed by Genencor along with a simple lineage diagram to show their relationship is also given in Appendix 3.

Strain GICC03278, the strain that produces chymosin B, has been modified from previous Genencor *T. reesei* strains by deletion of one gene and introduction of the *T. reesei* chaperonin gene placed under the expression signals of an endogenous *T. reesei* gene, along with the modification to have it produce chymosin B. These modifications do not change the safety profile of the established safe strain lineage.

7.2 Safety of Chymosin

Rennet from animal sources and chymosin from three recombinantly-derived microbial sources (*E. coli* K-12, *K. marxianus* var. *lactis* and *A. niger* var. *awamori*) are GRAS under FDA's food regulation 21 C.F.R. § 184.1685.¹⁶ Chymosin preparations from the three microbial sources were evaluated by JECFA, and an ADI not specified was established for all three.³⁵ Chymosin from these three microbial sources have also been evaluated by many other regulatory bodies around the world,

including those in the UK, Canada, Australia/New Zealand, and determined to be safe for the specified level of use.

As Dr. Pariza states in his June 19, 2007 opinion, “The *Bos taurus* chymosin B gene and its enzyme product (chymosin B) have a long history of safe use in cheese-making both in their original form (derived from calf stomach) and from production microorganisms expressing cloned *Bos Taurus* chymosin B gene into *T. reesei* genes. The methodology used to clone the *Bos Taurus* gene into *T. reesei* RL-P37 to produce the new production strain, *T. reesei* Pent CHY-Bip3 [also called GICC03278], did not involve insertion of antibiotic resistance markers or any other DNA fragments that could result in the synthesis of products that might present a safety concern. Viable production organisms are not present in the final chymosin B enzyme product.” (See Appendix 4.)

7.2.1 Allergenicity

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

Because exposure to enzymes is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually nil. In addition, the chymosin B preparation that is the subject of this notification is virtually identical to the other chymosins currently being used in the market, and Genencor is not aware of any documented allergic reactions to chymosin in the consumer population. Finally, chymosin is typically used in cheese products, which list enzymes on the product label.

7.2.2 Substantial Equivalence

Chymosin B produced by genetically modified *T. reesei*, the subject of this notification, is essentially identical to the *A. niger* var. *awamori* derived chymosin regulated under 21 C.F.R. § 184.1685. The specifications developed for the chymosin B enzyme meet or exceed standards set forth in FCC and FAO/WHO JECFA. Accordingly, Genencor maintains, as corroborated by Dr. Pariza (See Appendix 4), that Genencor’s chymosin B enzyme product is substantially equivalent to chymosin enzyme products currently in commerce.

7.3 Safety of the Manufacturing Process

The manufacturing process for the production of chymosin B 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, precipitation, dissolution, cellulose treatment, filtration and formulation, resulting in a liquid chymosin B enzyme preparation. The process, described in Section 4, is conducted in accordance with FDA's food CGMP's as set forth in 21 C.F.R. Part 110. The resultant product meets the general requirements for enzyme preparations of the FCC and WHO/JECFA.

7.4 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 *T. reesei* 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, Genencor, using the safe strain lineage concept of Pariza and Johnson (See Appendix 5), determined that no additional safety studies on chymosin B are warranted.

7.5 Identification of the NOAEL

Genencor has conducted a 90-day oral (gavage) study in rats (Scantox No. 60623) with another enzyme produced from a genetically modified strain of *T. reesei*, GICC03243, which is derived from the same production host and strain lineage (RL-P37) as used for chymosin B. In that study, a no observed adverse effect level ("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 chymosin B is through oral ingestion, selection of this NOAEL was deemed appropriate.

7.6 Human Exposure Assessment

Estimation of daily consumption of chymosin B was calculated based on its potential uses in major commodities (consumption data based on USDA-NASS Agricultural Statistics 2005; www.usda.gov/nass/pubs/agr05/).

Based on the analytical data conducted on chymosin B, the Total Organic Solids (“TOS”) for three lots of chymosin concentrate were determined to be 1.21%, 0.86%, and 0.82% with an average of 0.96%. Therefore, one ml of chymosin B contains 9.6 mg TOS. The recommended dose of chymosin B in a typical cheese making process is 0.1 ml/liter of milk (round-off from 0.09 ml/L milk) or 0.0001 ml chymosin B/ml of milk. The concentration of chymosin B expressed as TOS used in cheese making process is:

$$0.0001 \text{ ml chymosin/ml milk} \times 9.6 \text{ mg TOS/ml chymosin B} = 0.001 \text{ mg TOS/ml milk.}$$

As indicated in Section 6.3, approximately 10% of chymosin B will go into the cheese and 90% into the whey. Therefore, the use levels of chymosin B in cheese and whey are 0.0001 mg TOS/g cheese and 0.0009 mg TOS/g whey, respectively.

1. Cheese (all types excluding cottage cheese)
 - a. Annual consumption: 31.3 lbs or 14.20 kg
(1 lb = 0.454 kg)
 - b. Daily consumption: 38.9 g/d or 0.60 g/kg bw/day
(average human = 65 kg bw)
 - c. Dose of chymosin used: 0.0001 mg TOS/g cheese
 - d. Daily consumption of chymosin: 0.0001 mg TOS/g X 0.60 g/kg/d
0.00006 mg TOS/kg bw/day
2. Cottage cheese
 - a. Annual consumption: 2.70 lbs or 1.23 kg
 - b. Daily consumption: 3.36 g/d or 0.05 g/kg bw/day
 - c. Dose of Chymosin used: 0.0009 mg TOS/g cottage cheese
 - d. Daily consumption of Chymosin: 0.0009mg TOS X 0.05 g/kg bw
0.00005 mg TOS/kg bw/day
3. Whey protein
 - a. Annual consumption: 2.90 lbs or 1.32 kg
 - b. Daily consumption: 3.62 g/d or 0.06 g/kg bw/day
 - c. Dose of chymosin used: 0.0009 mg TOS/g whey
 - d. Daily consumption of chymosin: 0.0009 mg TOS X 0.06 g/kg
0.00005 mg TOS/kg bw/day

Maximum intake of Chymosin (expressed as TOS) from all commodities (cumulative):

$$0.00006 + 0.00005 + 0.00005 \text{ mg TOS/kg bw/day} = 0.00016 \text{ mg TOS/kg bw/day}$$

The maximum daily consumption of chymosin B from cheese (all types), cottage cheese (whey-cheese) and whey protein is 0.00016 mg TOS/kg bw/day under the scenario that (1) all above commodities are treated with chymosin B and (2) 100% of chymosin B is not removed during processing. Although it is expected that residues of a processing aid in the final products would be inactivated and/or negligible after processing, a 100% residue is used in this risk assessment to illustrate a worst-case-scenario.

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

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

Margin of safety = $\frac{35.81 \text{ mg TOS/kg bw/day}}{0.00016 \text{ mg TOS/kg bw/day}} = > 200,000$

7.7 Conclusion

The safety of chymosin B as a food processing aid in clotting or coagulation of milk during the manufacture of cheese is assessed using the toxicology data generated from another enzyme manufactured with a strain of *T reesei* from the same host organism lineage. Daily administration of the other enzyme for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at greater than 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day).

Even under a worst-case scenario where a person is consuming cheese, cottage-cheese and whey proteins that are 100% treated with chymosin B and 100% of the enzyme remains during processing (i.e., cumulative risk), this NOAEL still offers over 200,000 X fold margin of safety. Therefore, the use of chymosin B is not expected to result in adverse health effects.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *T reesei*, and enzyme preparations derived thereof, including cellulase, beta-glucanase, xylanase, acid fungal protease, 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)³⁵, as well as Genencor's own unpublished safety studies, support such a conclusion.

T reesei is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. In addition, the *T reesei* lineage used by Genencor has been demonstrated to be safe.

Chymosin, as regulated in 21 C.F.R. § 184.1685, is GRAS when produced by three other microbial sources, as discussed above. Based on TOS and extrapolation of the NOAEL, this chymosin B has a margin of safety of >200,000 times for its anticipated uses in cheese and whey.

Based on the publicly available scientific data from the literature and additional supporting data generated by Genencor, the company has concluded that bovine chymosin B from *T reesei* strain GICC03278 is safe and suitable for use in cheese manufacture and is 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 4).

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Appendix 1: Protein Sequence of Chymosin B

Appendix 2: Chymosin Manufacturing Process

Appendix 3: Strain Lineage Toxicology Test Summaries

Appendix 4: Pariza GRAS Assessment

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

Appendix I

Appendix 1: Protein Sequence of Chymosin B

The signal sequence or presequence is: mrelvllav falsqg

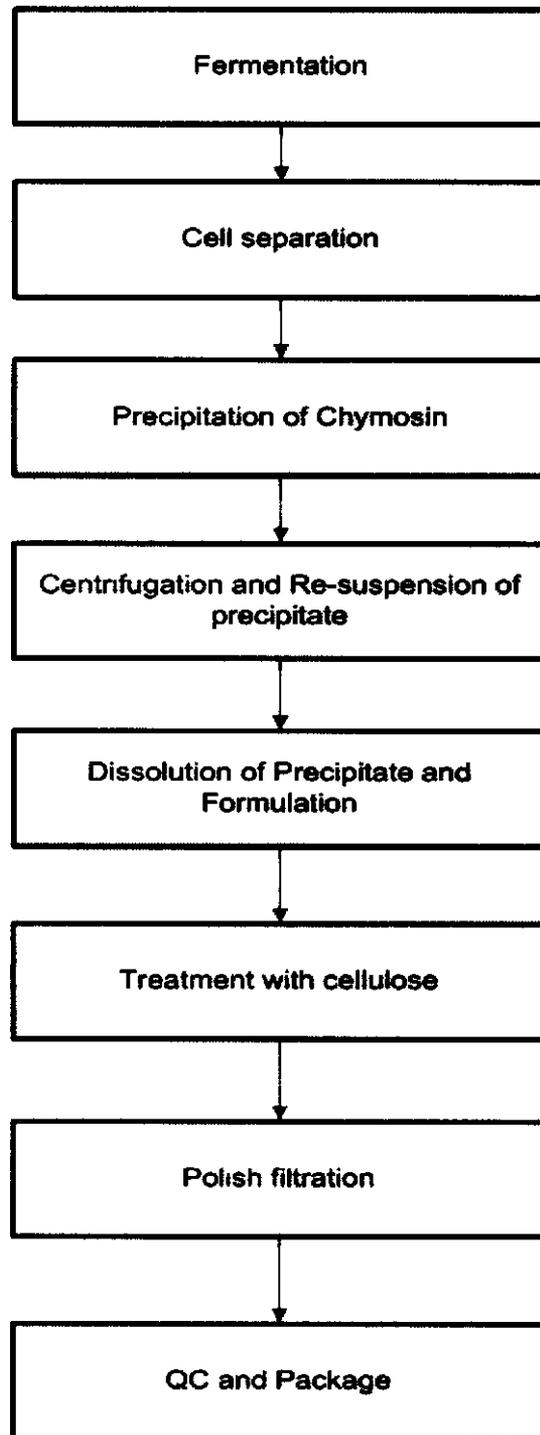
The prosequence is: acit riplykgksl rkalkehgll edflqkqqyg isskysgf

The mature chymosin B sequence is:

gevasvpltnyl dsqyfgkiyl gtppeqftvl fdtgssdfwv psiycksnac knhqrfdprk
sstfqnlgkp lsihygtgsm qgilgydtvt vsnivdiqqt vglstqepgd vftyaefdgi
lgmaypslas eysipvfdnm mnrhlvaqdl fsvymdrngq esmltlgaid psyytgsllhw
vpvtvqqywwq ftvdsvtisg vvvaceggcqq aildtgtskl vgpssdilni qqaigatqng
ygefdidcdn lsymptvvfe ingkmypltp saytsqdqgf ctsgfqsenh sqkwilgdvf
ireyysvfdr annlvglaka i

Appendix 2

Appendix 2:
Chymosin Manufacturing Process



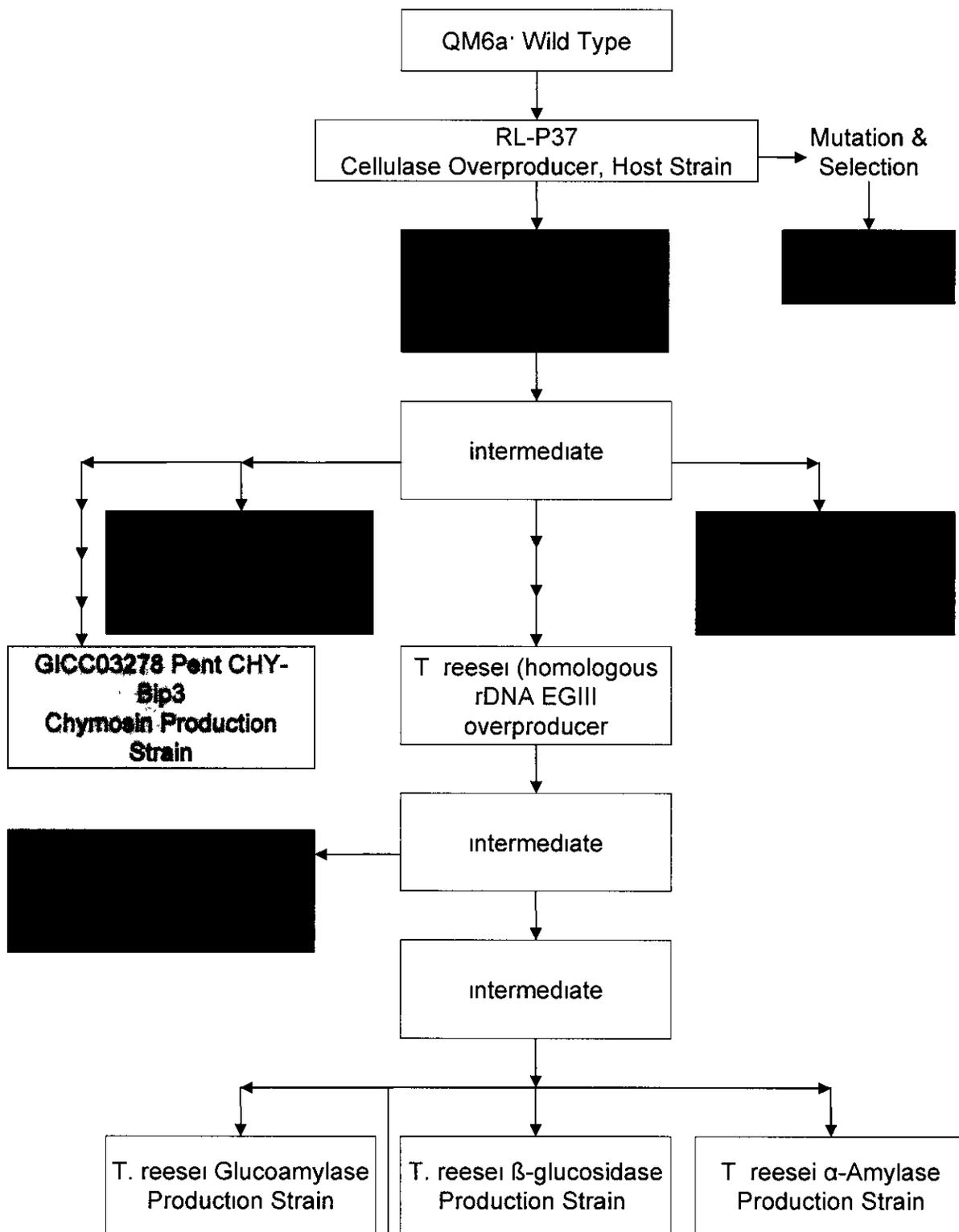
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Appendix 3

APPENDIX 3

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Appendix 3: Strain Lineage and Toxicology Test Summaries



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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. <i>T. reesei</i> A83 (traditionally modified)	<i>T. reesei</i> cellulase	Pathogenicity study, rats	Non-pathogenic, non-toxicogenic
		91-day subchronic study, rats	No adverse effect detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
II. <i>T. reesei</i> (homologous rDNA)	high pI xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, Chinese hamster ovary cells	Not clastogenic
III. <i>T. reesei</i> (homologous rDNA)	low pI xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
IV. <i>T. reesei</i> (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. <i>T. reesei</i> (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
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
VI. <i>T. reesei</i> (homologous rDNA)	Xylanase (protein engineered)	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
VII. <i>T. reesei</i> (homologous rDNA)	Acid Fungal Protease	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> 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)

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 2.2×10^7 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvrA* 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, ophthalmology, 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 *Escherischia coli* tester strain WP2 *uvrA* 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherischia coli* tester strain WP2 *uvrA* 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 pl xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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

A. 28-day oral study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase III (EG III) was given by gavage to groups of male and female Charles River rats at 0 (deionized water), 40, 200 or 1000 mg/kg/day for 28 consecutive days (Pharmakon, 1995).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* EG III. Based upon these findings, it was concluded that the treatment of male and female rats with EG III did not result in toxicity and a NOEL (No Observed Effect Level) was established at 1000 mg/kg/day.

B. Bacterial reverse mutation assay (Ames assay)

The test article, EG III, was examined for mutagenic activity in 5 strains of *Salmonella typhimurium* and *Escherichia coli* strain WP2uvrA 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, ophthalmology, 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherischia coli* tester strain WP2 *uvrA* in the presence and absence of metabolic activation (MDS Pharma, 2002). The assay was conducted in accordance with OECD Guideline 471 using the plate incorporation method (first assay) and treat and plate method (second assay).

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

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

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

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.0 to 3600 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 3600 µg/ml were selected for the chromosome aberration assay. In the chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of

this investigation, xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

VII. Acid Fungal Protease 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 35.81 mg 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, acid fungal protease (AFP) from *T. reesei* was tested in five strains of *Salmonella tyhimurium* (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 AFP 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, AFP 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 AFP 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 AFP concentrate both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, AFP concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation up to and including the maximum recommended dosage level of 5000 µg/ml.

D. Acute dermal irritation in rabbits

This study was conducted according to the method recommended in the OECD Guideline No. 404. In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application (Scantox, 2006).

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays. The mean score for skin edema and erythema was 0.0. AFP is classified as “not a skin irritant”.

E. Ocular irritation in rabbits

This study was conducted according to the method recommended in the OECD Guideline No. 405. In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded 1 and 24 hours later. The right eye served as control. After the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage at 48 and 72 hours after the treatment. A confirmatory test was conducted with 2 rabbits (Scantox, 2006).

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, no irritation was observed. The primary eye irritation score was 0.0. Under the conditions of this assay, AFP is classified as “not an eye irritant”.

F. 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 (Acute 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), AFP can be classified as non-hazardous (oral LD₅₀ > 2000 mg/kg bw).

Appendix 4

Appendix 4: Pariza GRAS Assessment

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

Michael W. Pariza, Member

June 19, 2007

Alice J. Caddow
Vice President, Regulatory
And Environmental Affairs
Genencor International, Inc.
925 Page Mill Road,
Palo Alto, CA 94304

Dear Ms. Caddow:

I have reviewed the information you provided on Genencor Danisco's Bovine chymosin B that is produced in *Trichoderma reesei* Pent CHY-Bip3, a recombinant strain derived from *T. reesei* RL-P37. In this evaluation I considered the biology of *T. reesei*, information available in the peer-reviewed scientific literature, and additional 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. *T. reesei* RL-P37, and enzyme preparations derived from this organism including cellulase, beta-glucanase, xylanase, acid fungal protease, alpha-amylase and glucoamylase, are well recognized as safe by qualified experts. Published literature, government laws and regulations for example FR 64:28658-28362; 1999, reviews by expert panels such as FAO/WHO JECFA (1992), well as Danisco's unpublished safety studies, support this conclusion. The new chymosin B production strain, *T. reesei* Pent CHY-Bip3, is derived from this safe strain lineage.

The *Bos taurus* chymosin B gene and its enzyme product (chymosin B) have a long history of safe use in cheese-making both in their original form (derived from calf

stomach) and from production microorganisms expressing cloned *Bos taurus* chymosin B genes. The methodology used to clone the *Bos taurus* chymosin B gene into *T. reesei* RL-P37 to produce the new production strain, *T. reesei* Pent CHY-Bip3, did not involve insertion of antibiotic resistance markers or any other DNA fragments that could result in the synthesis of products that might present a safety concern. Viable production organisms are not present in the final chymosin B enzyme product

The specifications you have developed for the chymosin B enzyme product are appropriate for a food grade enzyme. The product meets or exceeds standards set forth in Food Chemicals Codex and FAO/WHO JEFCA. I have also reviewed your recent production changes and agree with your assessment that they are appropriate for a food grade enzyme. Accordingly your new Genencor chymosin B enzyme product is substantially equivalent to chymosin enzyme products currently in commerce. Since the new Genencor chymosin B enzyme product will simply replace chymosin enzyme products currently in commerce, additional consumer exposure to chymosin is not anticipated as a result of this application.

Based on this information, I concur with the evaluation made by Genencor Danisco, that the new Genencor Danisco *T. reesei* Pent CHY-Bip3 production organism that expresses the *Bos taurus* chymosin B enzyme product is safe to use for the manufacture of food grade chymosin B. I further conclude that the chymosin B enzyme preparation manufactured using this organism by the process you described is GRAS for use as a food-processing ingredient.

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,

Michael W. Pariza
Wisconsin Distinguished Professor of
Food Microbiology and Toxicology
Member, Michael W. Pariza Consulting LLC

Appendix 5

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

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the Chymostar Supreme product 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 3;
3. **Issues relating to the introduced DNA are addressed:**
 - a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** – Yes, rDNA derived chymosins have been accepted as safe and used to make cheese since the early 1990s. Go to 3c;
 - c. **Is the test article free of transferable antibiotic resistance gene DNA?** – Yes, go to 3e;
 - e. **Is all other introduced DNA well characterized and free of attributes that would render it un-safe for constructing microorganisms to be used to produce food-grade products?** – Yes, go to 4;
4. **Is the introduced DNA randomly integrated into the chromosome?** – Yes, inserted DNA is well characterized, but complete characterization of the location of all insertions is not possible 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 methods that were employed?** – In general, yes except as noted in 4. Go to 6;
6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** – Yes, *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing; Genencor has conducted toxicology testing on 7 different products derived from this lineage and all were determined to be safe for their intended food uses; Accept.

Conclusion: Article is accepted



Brown, Anna Marie

Subject: Brown, Anna Marie
Chymosin

From: Alice Caddow [<mailto:alice.caddow@danisco.com>]
Sent: Tuesday, August 14, 2007 5:41 PM
To: Tarantino, Laura M
Subject: Chymosin GRAS Notice

Dear Dr Tarantino

The GRAS Notice for chymosin submitted by Genencor on July 20, 2007 includes two incorrect appendices, unfortunately. Insertion of the correct appendices will change the page numbers after appendix 2. To correct this error, should we submit just the corrected pages or 3 new copies of the submission? Should we withdraw the current one to make these corrections? We should have the correct version ready by Wednesday or Thursday, August 15, 16. I apologize profusely for this error.

Thank you in advance for your help in resolving our mistake.

Sincerely,

Alice
Alice J. Caddow
Vice President of Regulatory
and Environmental Affairs
Genencor, A Danisco Division
25 Page Mill Road
Palo Alto, CA 94304-1013
Phone 1-650-846-7557
Fax 1-650-845-6505
email alice.caddow@danisco.com

Brown, Anna Marie

Subject: Chymosin

From: Alice Caddow [<mailto:alice.caddow@danisco.com>]

Sent: Tuesday, August 14, 2007 6:47 PM

To: Gaynor, Paulette M

Subject: Fw: Chymosin GRAS Notice

Dear Dr. Gaynor,

On further investigation, we also discovered that some of the pages in the submission reference incorrect Appendices numbers, so it will probably be easier to send you three new copies. Please let me know your preference. Again, my apologies for the errors.

Sincerely,

Alice J. Caddow

Brown, Anna Marie

From: Alice Caddow [alice.caddow@danisco.com]
Sent: Wednesday, August 22, 2007 1:30 PM
To: Brown, Anna Marie
Subject: Chymosin GRAS Notice

Dear Dr. Brown,

We are sending the 3 corrected copies of this notice to your attention today to arrive tomorrow. Should you have any questions, please do not hesitate to contact me.

Sincerely,

Alice J. Caddow
Vice President of Regulatory
and Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
Phone: 1-650-846-7557
Fax: 1-650-845-6505
email: alice.caddow@danisco.com

000046

9/17/2007



August 22, 2007

Dr. Anna Marie Brown, CSO
Office of Food Additive Safety, HFS-820
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Brown:

As explained in my emails, we discovered that we mistakenly sent an earlier, incorrect version of the GRAS Notice for chymosin that was sent to the FDA on July 20, 2007. Enclosed herein are three copies of the corrected GRAS Notice which we ask you to use for review. We apologize for any problems this mistake may cause you or other reviewers.

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)



**Chymosin Enzyme Preparation from *Trichoderma reesei*
expressing the Chymosin B gene from *Bos Taurus*
is Generally Recognized As Safe**

Notification Submitted by Genencor, a Danisco Division

August 22, 2007

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1. GENERAL INTRODUCTION

Chymostar Supreme is the Danisco trade name used for the chymosin enzyme preparation produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding chymosin B from *Bos taurus*. DNA encoding the prochymosin portion of the chymosin protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence and, subsequently, inserted into *T. reesei*.

The enzyme is to be used in the food industry as a processing aid for the manufacture of cheese.

The enzyme is an aspartic endopeptidase (EC 3.4.23.4) which has broad specificity similar to that of pepsin A and clots milk by cleavage of a single 105-Ser-Phe-|-Met-Ala-108 bond in the kappa-chain of casein. It is added to milk to form curds and whey, the first step in cheese manufacture. The curds are then processed further to form cheese and the whey is used as a protein source in many food applications.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor has determined that its chymosin B enzyme preparation from a modified strain of *T. reesei* expressing the chymosin B gene from *Bos Taurus* 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 a chymosin B enzyme preparation produced by a *T. reesei* host, which has been modified to express a gene encoding chymosin B from *Bos taurus*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for food use.^{1,2} The safety of the production organism for this chymosin B, *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. Data showing this chymosin B to be substantially equivalent^{5,7,8-11} to naturally occurring chymosin B is provided in Section 3. 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 Name and Address of Notifier

Genencor, A Danisco Division
200 Meridian Centre Boulevard
Rochester, NY 14618-3916

1.2 Common or Usual Name of Substance

Chymosin enzyme preparation from *T. reesei* expressing the gene encoding chymosin B enzyme from *Bos taurus*

1.3 Applicable Conditions of Use

The chymosin preparation is intended for use in the cheese making industry for milk coagulation during cheese making. The enzyme preparation is used at minimum levels to achieve the desired effect and according to requirements of normal production following current Good Manufacturing Practices. It is expected that cheese produced using this chymosin will be consumed by the general population.

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 which supports this GRAS determination is enclosed with this letter. 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 of the chymosin B enzyme preparation is *T. reesei* strain GICC03278. It is derived by recombinant DNA methods from strain RL-P37 (see Section 2.2). This genetically modified production microorganism complies with the Organization for Economic Co-operation and Development (“OECD”) criteria for Good Industrial Large Scale Practice (“GILSP”) microorganisms.¹² It also meets the criteria for a safe production microorganism as described by Pariza and Johnson¹ and several expert groups.²⁻⁸ It contains the synthetic chymosin B gene under the regulation of a native *T. reesei* promoter and terminator along with a selectable marker from *A. nidulans* and a *T. reesei* chaperonin gene under the regulation of a different native *T. reesei* promoter and terminator to assist in the excretion of chymosin B from the microorganism.

2.2 Recipient Microorganism

The recipient microorganism used in the construction of the chymosin production strain is a strain of the well-known industrial production strain, *T. reesei*. It is derived from strain RL-P37 obtained from Dr. Montenecourt; the derivation and characterization of strain RL-P37 has been published.¹³ *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina*.^{14,15} Genencor has optimized this strain for the production of enzyme proteins through several gene deletions; the gene deletions did not leave any foreign DNA in the *T. reesei* recipient.

2.3 Chymosin Expression Vector

The transforming DNA consisted of two expression cassettes:

- One expression cassette containing the prochymosin B DNA fused to a *T. reesei* promoter and terminator to control expression and using a well-characterized and recognized selectable marker from *A. nidulans*; and
- The second expression cassette containing a *T. reesei* chaperonin that resides in the endoplasmic reticulum and is involved in folding of nascent secreted proteins fused to a different *T. reesei* promoter and terminator to control expression.

DNA encoding the prochymosin portion of the chymosin B protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence to maximize the gene's expression in the *T. reesei* recipient. 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 presence of the introduced DNA sequences was determined by Southern blot analysis to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism.³⁻⁸ The analysis confirms that the introduced DNA is stably integrated into the *T. reesei* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable.¹²

2.5 Antibiotic Resistance Gene

The DNA used for transforming the *T. reesei* host strain does not contain any antibiotic resistance genes.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product. The production organism does not end up in food, and therefore, the first step in the safety assessment as described by the IFBC³ is satisfactorily addressed.

An antibiotic resistance gene was not used in this construction, therefore, there is none present in the enzyme preparation. The production strain does not carry any vectors. As no vector sequences are present in the final strain, the transfer frequency of the integrated expression cassettes is the same as for any other chromosomal sequence. The expression cassettes are completely derived from fungal and synthetic DNA.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Key enzyme and protein chemical characteristics of the chymosin are:

Classification	aspartic endopeptidase
IUB Nomenclature	chymosin
IUB Number:	3.4.23.4
CAS Number:	9001-98-3
EINECS Number:	232-645-0
Reaction catalyzed:	Broad specificity similar to that of pepsin A. Clots milk by cleavage of a single 105-Ser-Phe- -Met-Ala-108 bond in kappa-chain of casein.
Molecular weight:	Approximately 35 kDa
Amino Acid Sequence:	The total nucleotide and amino acid sequences have been determined.

Native chymosin (commercial extracts derived from animals containing the active enzyme rennin) and chymosin preparations (derived from fermentation from other bioengineered microbial strains (*Escherichia coli*, *Kluyveromyces marxianus* var. *lactis*, and *Aspergillus niger* var. *awamori*)) are the subject of GRAS affirmation regulation 21 C.F.R. § 184.1685.¹⁶ The regulation states that the enzyme may be used in food with no limitation other than current good manufacturing practices, and it affirms that this ingredient is GRAS as a direct human food ingredient when used as a processing aid (21 C.F.R. § 170.3(o)(24)) or stabilizer and thickener (21 C.F.R. § 170.3(o)(28)) at levels not to exceed current good manufacturing practice in cheeses (21 C.F.R. § 170.3(n)(5)), frozen dairy desserts and mixes (21 C.F.R. § 170.3(n)(20)), gelatins, puddings, and fillings (21 C.F.R. § 170.3(n)(22)), and milk products (21 C.F.R. § 170.3(n)(31)).

3.2 Amino Acid Sequence

The amino acid sequence of Chymostar Supreme chymosin B is identical to the amino acid sequence of native chymosin B. The amino acid sequence of chymosin B is shown in Appendix I.

3.3 Comparison to other chymosins

Bovine chymosin B is well characterized and is an aspartic protease used as a milk coagulant in cheese-making. It is synthesized in the mucosa of the abomasum (fourth stomach) of unweaned calves as preprochymosin B. Preprochymosin B consists of a 16 amino acid pre-region or secretion signal sequence, a propeptide of 42 amino acids, and the mature chymosin B protein of 323 amino acids. The pre-region directs secretion of the protein and is removed by proteolysis as the protein passes through the membrane from the cytoplasm into the lumen of the endoplasmic reticulum. Prochymosin B is inactive and is secreted to the extracellular space. At low pH (pH 4-5) the propeptide is autocatalytically cleaved to release mature, active chymosin B.

The Genencor chymosin B is identical in sequence to the native chymosin B sequence as specified in the National Center for Biotechnology Information (NCBI) or Swiss-Prot protein sequence databases under accession number P00794. There is a single amino acid difference between the Genencor chymosin B sequence and the sequence of the chymosin B produced by *Aspergillus niger* var. *awamori*, which is the subject of the aforementioned regulation, 21 C.F.R. § 184.1685. This one amino acid difference should not be considered significant as there are different sequences of chymosin B deposited at NCBI, most likely due to different alleles being cloned. These different sequences and their relationship to the P00794 sequence are summarized in the NCBI database entry for P00794.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the chymosin enzyme preparation which follows standard industry practice.¹⁷⁻¹⁹ 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 the chymosin enzyme concentrate are standard ingredients used in the enzyme industry.¹⁷⁻¹⁹ All the raw

materials conform to the specifications of the Food Chemicals Codex, 5th edition, 2003 (“FCC”),²⁰ except for those raw materials which 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. Genencor uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and quality specifications are verified upon receipt. The antifoams used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the production process is <1%.

4.2 Fermentation Process

The chymosin is manufactured by submerged 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 both macroscopically and microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient medium or in an enrichment shake flask followed by a 24-72 hour incubation period.

The fermentation is declared as ‘contaminated’ if infection is observed in 3 successive media plates at a minimum interval of 24 hours.

If a fermentation 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 purification and formulation processes.

4.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment – pH adjustment and cooling
2. Primary separation – rotary vacuum drum filtration
3. Concentration – ultrafiltration and precipitation/centrifugation
4. Dissolution – of precipitate and treatment with cellulose
5. Polish filtration – for removal of residual production strain organisms and as general precaution against microbial degradation

4.3.2 Formulation and Standardization Process

The product is formulated with sodium chloride, propylene glycol, methionine and sodium benzoate and standardized according to the product specification to the right concentration. Caramel color may be added.

4.4 Quality Control of Finished Product

The final chymosin preparation 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 (“JECFA”) in 2006 and the FCC. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative composition

The chymosin enzyme preparation has the following typical composition:

Enzyme: 0.24 -0.30% active protein
Sodium benzoate: 0.26-0.35% (w/w)
Sodium chloride: 10-15% (w/w)
Propylene glycol: 2.5-3.5% (w/w)
Methionine: 0.1-0.15% (w/w)
pH adjustment to 5.8-6.25 with 10% sodium hydroxide
Water: ~80-87%.

5.2 Specifications

The chymosin enzyme preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC. See FCC (2003)²⁰ at 149. In addition, the chymosin enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA.²¹

The following specifications have been established for the chymosin enzyme preparation:

(See next page)

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Chymosin B (International Milk Clotting Units)	IDF 157A	International IDF Standard 157A:1997 – “Determination of total milk-clotting activity”	IMCU/ml 600-750
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - “Microbiology - General guidance for the enumeration of micro-organisms - colony count technique at 30°C” and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - “General guidance for the enumeration of coliforms - colony count technique” and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
<i>E coli</i>	819V	ISO 7251 - Microbiology - “General Guidance for Enumeration of Presumptive <i>Escherichia coli</i> - Most Probable Number Technique” and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g

Property	Method Number	Reference Method	Specification
MICROBIOLOGICAL ANALYSIS (Continued)			
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Production strain	892V	Genencor Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Heavy Metals, as Pb	603W	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests Test solutions, and other reference materials, 1983, 2 nd revision	Less than 30 mg/kg
Arsenic	603W-AS	Same as Heavy Metals as Lead	Less than 3 mg/kg
Cadmium	603W-CD	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Mercury	603W-HG	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Lead	603W-PB	Same as Heavy Metals as Lead	Less than 5 mg/kg
Mycotoxins	604W	Patterson & Roberts, Assoc. of Anal. Chem. (vol. 62, no. 6, 1979)	Negative by test

Heavy metals, lead, arsenic, cadmium, mercury, antibacterial activity, and mycotoxins are analyzed at regular intervals. Activity and microbial specifications are analyzed on every lot.

The lead, Coliforms, and Salmonella specifications meet FCC and JECFA requirements for enzyme preparations cited above. The *E. coli*, antibacterial activity and mycotoxin specifications meet JECFA requirements and are not included in FCC (although FCC mentions mycotoxins, but has not established tolerances). The production microorganism specification is a Genencor specification and is not mentioned in FCC or JECFA.

The chymosin activity assay measures the milk clotting ability of the enzyme in International Milk Clotting Units according to a standard assay from the International Dairy Federation.

6. APPLICATION

6.1 Mode of Action

As noted above, chymosin B causes endohydrolysis of peptide linkages in proteins.

Chymosin B will be used for clotting or coagulation of milk during the manufacture of cheese, just as the other commercial chymosins already on the market are used. The resultant curds will be used to make cheese. The resultant whey can be concentrated to meet various specifications, or it can be separated into the protein and carbohydrate (i.e., lactose) fractions and used as a food ingredient. Whey proteins are often used in nutritional supplements, and low pH beverage products and the lactose is used as a carbohydrate source in many food products.

6.2 Use Levels

In a typical cheese-making process, the recommended dosage for this chymosin B is 50 imcu (approximately 0.06-0.09 ml) of product per liter of milk. This is the same or lower level of use as the use level for chymosin preparations that are the subject of 21 C.F.R. § 184.1685.

6.3 Enzyme Residues in the Final Foods

As with other chymosins, approximately 10% will go into the cheese and 90% into the whey. The 90% in the whey will tend to partition into the protein portion rather than the carbohydrate, or lactose, portion. Depending upon the use of the whey or whey fraction, the chymosin may be inactivated during the food processing steps. The fate of this chymosin is identical to those already in commerce. The residue level is

assumed to be the same as the residue level for the chymosin preparations that are the subject of 21 C.F.R. § 184.1685.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism is the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food.² If the organism is nontoxic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using procedures that adhere to FDA's food CGMPs under 21 C.F.R. Part 110, are safe to consume.³ Pariza and Foster² define a nontoxic 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 nonpathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *T. reesei* meets these criteria for nontoxigenicity and nonpathogenicity.

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 that *T. reesei* be placed into synonymy with *T. longibrachiatum*.²² Later however, there subsequently appeared evidence that the two species were not identical.²³ The proposal by Kuhls et al.¹⁴ 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, *T. reesei*, *T. longibrachiatum*, and *H. jecorina* may appear in different documents, but they refer to essentially the same microorganism species.

A review of the literature search on the organism (1972-2006) uncovered no reports that implicate *T. reesei* in connection with a disease, 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 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.

Bruckner and Graf reported the isolation from *T. reesei* strain QM 9414 (a different strain than the strain producing the chymosin that is the subject of this notification) of a peptaibol compound that exhibited antibiotic activity.²⁴ Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains.²⁵ 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, has been a safe producer of commercial cellulase enzyme preparations for food applications.²⁶ 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.²⁶, Blumenthal²⁷ and Olemska-Beer et al.²⁸ 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 paper¹ and various strains have been approved to produce commercial enzyme products internationally, for example, in Canada²⁹, the United States³⁰, France³¹, Australia/New Zealand³², China (MOH 1996)³³, and Japan³⁴. In addition, JECFA has reviewed cellulase from *T. reesei* and set an ADI of not specified for the enzyme.³⁵

The production organism of the chymosin B enzyme preparation, the subject of this submission, is *T. reesei* strain GICC03278. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to express the bovine chymosin B 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.¹³ 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 (see Appendix 3). One of these strains, modified to overexpress an endoglucanase enzyme, was tested for pathogenicity and toxicogenicity in an acute intraperitoneal study in rats and determined to be nonpathogenic and nontoxicogenic. Another study on strain A83, also derived from RL-P37 through mutation and selection, also determined the strain to be nonpathogenic and nontoxicogenic (see Appendix 3 for a summary of the toxicity data on these *T. reesei* strains and others). As Dr. Pariza states in his June 19, 2007 opinion, “*T. reesei* RL-P37, and enzyme preparations derived from this organism including cellulase, beta-glucanase, xylanase, acid fungal protease, alpha-amylase and glucoamylase, are well recognized as safe by qualified experts. 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 Genencor’s unpublished safety studies, support this conclusion. The new chymosin B production strain, *T. reesei* Pent CHY-Bip3 [also labeled GICC03278] is derived from this safe strain lineage.” (See Appendix 4.)

In accordance with the Pariza opinion, Genencor found there was no need to conduct any additional testing of the GICC03278 strain beyond that which was already performed (see Appendix 5).

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 chymosin B, and the GIGC03278 strain is non-pathogenic and non-toxicogenic.

7.1.1 Safety of the Donor Source

The donor organism for the prochymosin B gene used in construction of the new microorganism *T. reesei* GICC03278 was, indirectly, *Bos taurus*. The amino acid sequence of an allelic form (the B form) of bovine preprochymosin is available in the NCBI or Swiss-Prot protein sequence databases under accession number P00794. DNA encoding the prochymosin portion of the protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence. Therefore, the gene for chymosin and the resultant product, chymosin, are recognized as safe.

7.1.2 Safety of the Strain Lineage

QM6a-derived strains account for nearly all industrially produced (by fermentation) cellulase in the world.²⁴ The derivation and characterization of strain RLP-37 has been published by Sheir-Neiss and Montencourt¹³, which Genencor used to produce cellulases for over fifteen years. Genencor has developed many production strains from it using recombinant DNA techniques. One of these strains, modified to overexpress an endoglucanase enzyme, has been tested for pathogenicity and toxicogenicity in an acute intraperitoneal study in rats and was determined to be nonpathogenic and nontoxicogenic. Another strain A83, also derived from RL-P37 through mutation and selection, was also determined to be nonpathogenic and nontoxicogenic. See Appendix 3 for a summary of the toxicity data. Genencor established that all of the products produced by the QM6a-derived strains are safe for their intended uses. The toxicology testing conducted on the enzymes developed by Genencor along with a simple lineage diagram to show their relationship is also given in Appendix 3.

Strain GICC03278, the strain that produces chymosin B, has been modified from previous Genencor *T. reesei* strains by deletion of one gene and introduction of the *T. reesei* chaperonin gene placed under the expression signals of an endogenous *T. reesei* gene, along with the modification to have it produce chymosin B. These modifications do not change the safety profile of the established safe strain lineage.

7.2 Safety of Chymosin

Rennet from animal sources and chymosin from three recombinantly-derived microbial sources (*E. coli* K-12, *K. marxianus* var. *lactis* and *A. niger* var. *awamori*) are GRAS under FDA's food regulation 21 C.F.R. § 184.1685.¹⁶ Chymosin preparations from the three microbial sources were evaluated by JECFA, and an ADI not specified was established for all three.³⁵ Chymosin from these three microbial sources have also been evaluated by many other regulatory bodies around the world,

including those in the UK, Canada, Australia/New Zealand, and determined to be safe for the specified level of use.

As Dr. Pariza states in his June 19, 2007 opinion, “The *Bos taurus* chymosin B gene and its enzyme product (chymosin B) have a long history of safe use in cheese-making both in their original form (derived from calf stomach) and from production microorganisms expressing cloned *Bos Taurus* chymosin B gene into *T. reesei* genes. The methodology used to clone the *Bos Taurus* gene into *T. reesei* RL-P37 to produce the new production strain, *T. reesei* Pent CHY-Bip3 [also called GICC03278], did not involve insertion of antibiotic resistance markers or any other DNA fragments that could result in the synthesis of products that might present a safety concern. Viable production organisms are not present in the final chymosin B enzyme product.” (See Appendix 4.)

7.2.1 Allergenicity

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

Because exposure to enzymes is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually nil. In addition, the chymosin B preparation that is the subject of this notification is virtually identical to the other chymosins currently being used in the market, and Genencor is not aware of any documented allergic reactions to chymosin in the consumer population. Finally, chymosin is typically used in cheese products, which list enzymes on the product label.

7.2.2 Substantial Equivalence

Chymosin B produced by genetically modified *T. reesei*, the subject of this notification, is essentially identical to the *A. niger* var. *awamori* derived chymosin regulated under 21 C.F.R. § 184.1685. The specifications developed for the chymosin B enzyme meet or exceed standards set forth in FCC and FAO/WHO JECFA. Accordingly, Genencor maintains, as corroborated by Dr. Pariza (See Appendix 4), that Genencor’s chymosin B enzyme product is substantially equivalent to chymosin enzyme products currently in commerce.

7.3 Safety of the Manufacturing Process

The manufacturing process for the production of chymosin B 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, precipitation, dissolution, cellulose treatment, filtration and formulation, resulting in a liquid chymosin B enzyme preparation. The process, described in Section 4, is conducted in accordance with FDA's food CGMP's as set forth in 21 C.F.R. Part 110. The resultant product meets the general requirements for enzyme preparations of the FCC and WHO/JECFA.

7.4 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 *T. reesei* 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, Genencor, using the safe strain lineage concept of Pariza and Johnson (See Appendix 5), determined that no additional safety studies on chymosin B are warranted.

7.5 Identification of the NOAEL

Genencor has conducted a 90-day oral (gavage) study in rats (Scantox No. 60623) with another enzyme produced from a genetically modified strain of *T. reesei*, GICC03243, which is derived from the same production host and strain lineage (RL-P37) as used for chymosin B. In that study, a no observed adverse effect level ("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 chymosin B is through oral ingestion, selection of this NOAEL was deemed appropriate.

7.6 Human Exposure Assessment

Estimation of daily consumption of chymosin B was calculated based on its potential uses in major commodities (consumption data based on USDA-NASS Agricultural Statistics 2005; www.usda.gov/nass/pubs/agr05/).

Based on the analytical data conducted on chymosin B, the Total Organic Solids (“TOS”) for three lots of chymosin concentrate were determined to be 1.21%, 0.86%, and 0.82% with an average of 0.96%. Therefore, one ml of chymosin B contains 9.6 mg TOS. The recommended dose of chymosin B in a typical cheese making process is 0.1 ml/liter of milk (round-off from 0.09 ml/L milk) or 0.0001 ml chymosin B/ml of milk. The concentration of chymosin B expressed as TOS used in cheese making process is:

$$0.0001 \text{ ml chymosin/ml milk} \times 9.6 \text{ mg TOS/ml chymosin B} = 0.001 \text{ mg TOS/ml milk.}$$

As indicated in Section 6.3, approximately 10% of chymosin B will go into the cheese and 90% into the whey. Therefore, the use levels of chymosin B in cheese and whey are 0.0001 mg TOS/g cheese and 0.0009 mg TOS/g whey, respectively.

1. Cheese (all types excluding cottage cheese)
 - a. Annual consumption: 31.3 lbs or 14.20 kg
(1 lb = 0.454 kg)
 - b. Daily consumption: 38.9 g/d or 0.60 g/kg bw/day
(average human = 65 kg bw)
 - c. Dose of chymosin used: 0.0001 mg TOS/g cheese
 - d. Daily consumption of chymosin: 0.0001 mg TOS/g X 0.60 g/kg/d
0.00006 mg TOS/kg bw/day
2. Cottage cheese
 - a. Annual consumption: 2.70 lbs or 1.23 kg
 - b. Daily consumption: 3.36 g/d or 0.05 g/kg bw/day
 - c. Dose of Chymosin used: 0.0009 mg TOS/g cottage cheese
 - d. Daily consumption of Chymosin: 0.0009mg TOS X 0.05 g/kg bw
0.00005 mg TOS/kg bw/day
3. Whey protein
 - a. Annual consumption: 2.90 lbs or 1.32 kg
 - b. Daily consumption: 3.62 g/d or 0.06 g/kg bw/day
 - c. Dose of chymosin used: 0.0009 mg TOS/g whey
 - d. Daily consumption of chymosin: 0.0009 mg TOS X 0.06 g/kg
0.00005 mg TOS/kg bw/day

Maximum intake of Chymosin (expressed as TOS) from all commodities (cumulative):

$$0.00006 + 0.00005 + 0.00005 \text{ mg TOS/kg bw/day} = 0.00016 \text{ mg TOS/kg bw/day}$$

The maximum daily consumption of chymosin B from cheese (all types), cottage cheese (whey-cheese) and whey protein is 0.00016 mg TOS/kg bw/day under the scenario that (1) all above commodities are treated with chymosin B and (2) 100% of chymosin B is not removed during processing. Although it is expected that residues of a processing aid in the final products would be inactivated and/or negligible after processing, a 100% residue is used in this risk assessment to illustrate a worst-case-scenario.

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

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

Margin of safety = $\frac{35.81 \text{ mg TOS/kg bw/day}}{0.00016 \text{ mg TOS/kg bw/day}} = > 200,000$

7.7 Conclusion

The safety of chymosin B as a food processing aid in clotting or coagulation of milk during the manufacture of cheese is assessed using the toxicology data generated from another enzyme manufactured with a strain of *T reesei* from the same host organism lineage. Daily administration of the other enzyme for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at greater than 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day).

Even under a worst-case scenario where a person is consuming cheese, cottage-cheese and whey proteins that are 100% treated with chymosin B and 100% of the enzyme remains during processing (i.e., cumulative risk), this NOAEL still offers over 200,000 X fold margin of safety. Therefore, the use of chymosin B is not expected to result in adverse health effects.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *T reesei*, and enzyme preparations derived thereof, including cellulase, beta-glucanase, xylanase, acid fungal protease, 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)³⁵, as well as Genencor's own unpublished safety studies, support such a conclusion.

T reesei is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. In addition, the *T reesei* lineage used by Genencor has been demonstrated to be safe.

Chymosin, as regulated in 21 C.F.R. § 184.1685, is GRAS when produced by three other microbial sources, as discussed above. Based on TOS and extrapolation of the NOAEL, this chymosin B has a margin of safety of >200,000 times for its anticipated uses in cheese and whey.

Based on the publicly available scientific data from the literature and additional supporting data generated by Genencor, the company has concluded that bovine chymosin B from *T reesei* strain GICC03278 is safe and suitable for use in cheese manufacture and is 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 4).

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10. LIST OF APPENDICES

Appendix 1: Protein Sequence of Chymosin B

Appendix 2: Chymosin Manufacturing Process

Appendix 3: Strain Lineage Toxicology Test Summaries

Appendix 4: Pariza GRAS Assessment

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

Appendix I

Appendix 1: Protein Sequence of Chymosin B

The signal sequence or presequence is: mrelvllav falsqg

The prosequence is: acit riplykgksl rkalkehgll edflqkqqyg isskysgf

The mature chymosin B sequence is:

gevasvpltnyl dsqyfgkiyl gtppeqftvl fdtgssdfwv psiycksnac knhqrfdprk

sstfqnlgkp lsihygtgsm qgilgydtvt vsnivdiqqt vglstqepgd vftyaefdgi

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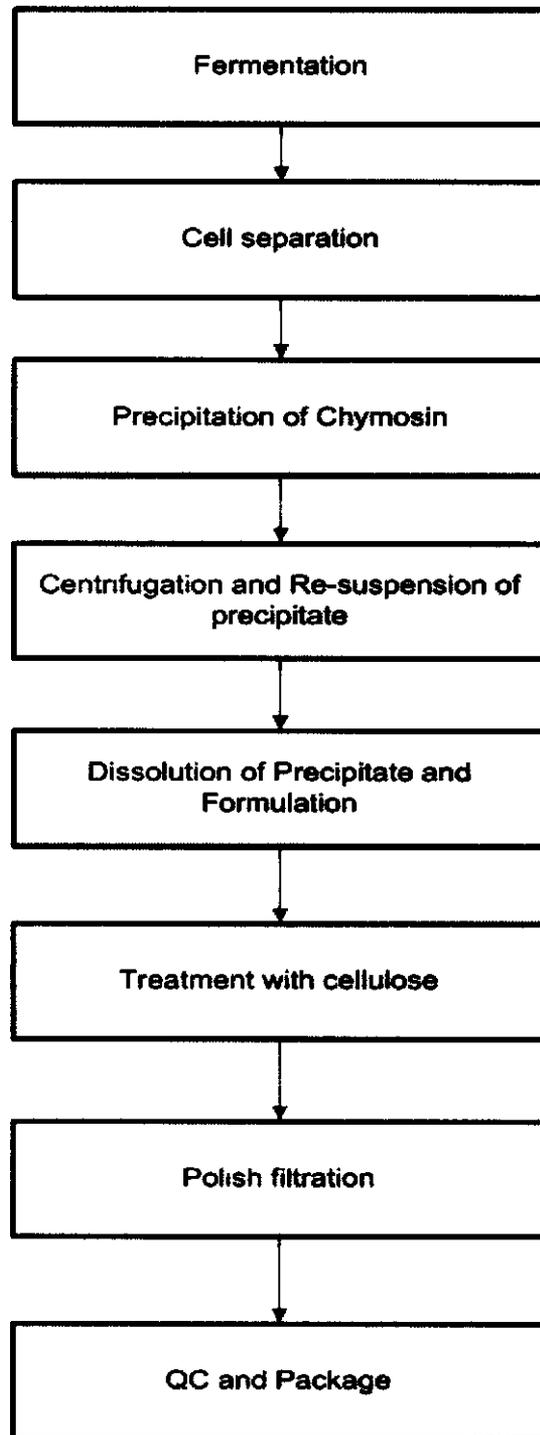
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ireyysvfdr annlvglaka i

Appendix 2

Appendix 2:
Chymosin Manufacturing Process



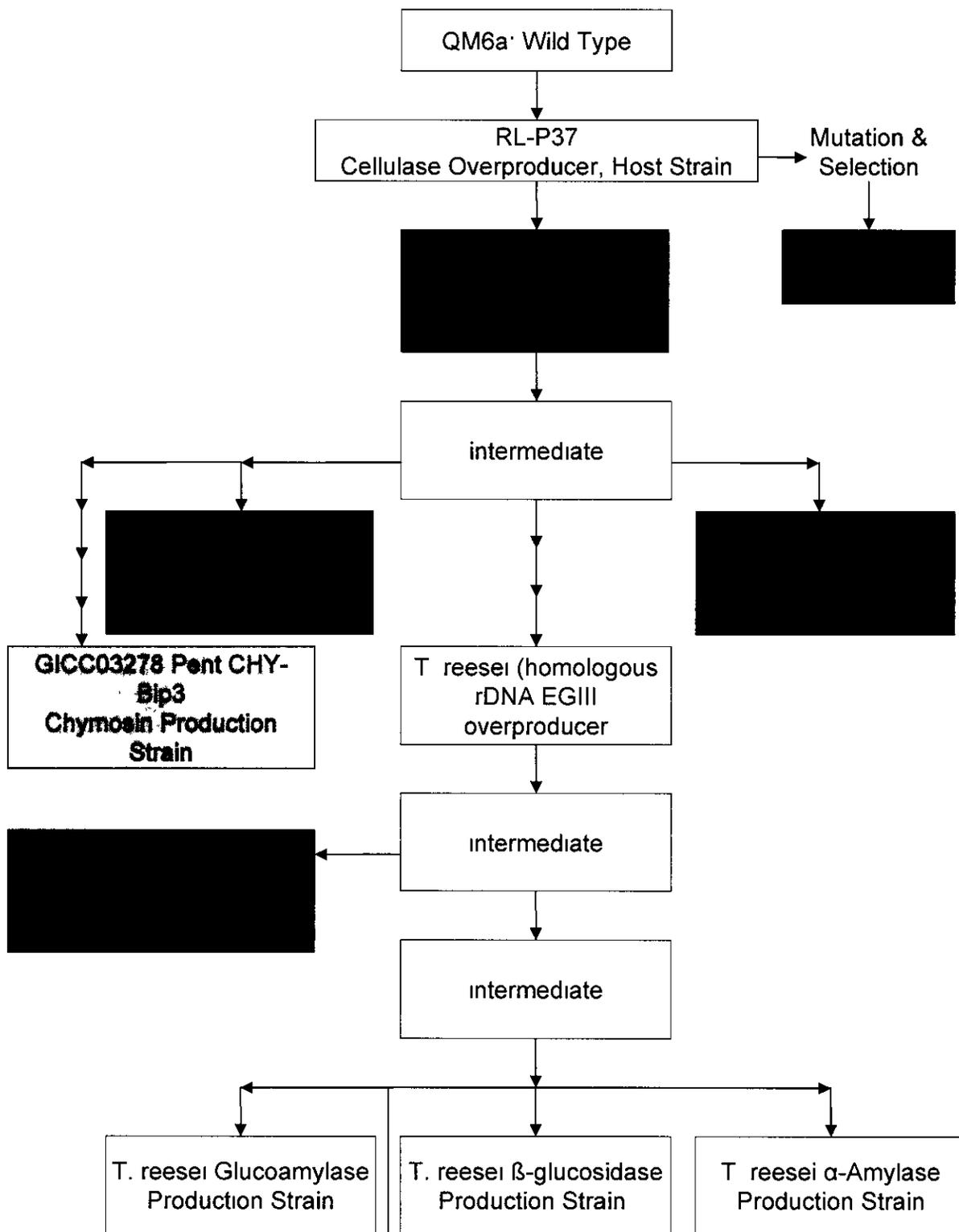
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Appendix 3

APPENDIX 3

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Appendix 3: Strain Lineage and Toxicology Test Summaries



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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. <i>T. reesei</i> A83 (traditionally modified)	<i>T. reesei</i> cellulase	Pathogenicity study, rats	Non-pathogenic, non-toxicogenic
		91-day subchronic study, rats	No adverse effect detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
II. <i>T. reesei</i> (homologous rDNA)	high pI xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, Chinese hamster ovary cells	Not clastogenic
III. <i>T. reesei</i> (homologous rDNA)	low pI xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
IV. <i>T. reesei</i> (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. <i>T. reesei</i> (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
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
VI. <i>T. reesei</i> (homologous rDNA)	Xylanase (protein engineered)	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		<i>In vitro</i> chromosome assay, human lymphocytes	Not clastogenic
		VII. <i>T. reesei</i> (homologous rDNA)	Acid Fungal Protease
Bacterial reverse mutation assay	Not mutagenic		
<i>In vitro</i> 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)		

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 2.2×10^7 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvrA* 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherischia coli* tester strain WP2 *uvrA* 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherischia coli* tester strain WP2 *uvrA* 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 pl xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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

A. 28-day oral study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase III (EG III) was given by gavage to groups of male and female Charles River rats at 0 (deionized water), 40, 200 or 1000 mg/kg/day for 28 consecutive days (Pharmakon, 1995).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* EG III. Based upon these findings, it was concluded that the treatment of male and female rats with EG III did not result in toxicity and a NOEL (No Observed Effect Level) was established at 1000 mg/kg/day.

B. Bacterial reverse mutation assay (Ames assay)

The test article, EG III, was examined for mutagenic activity in 5 strains of *Salmonella typhimurium* and *Escherichia coli* strain WP2uvrA 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, ophthalmology, 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, ophthalmology, 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 tyhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherischia coli* tester strain WP2 *uvrA* in the presence and absence of metabolic activation (MDS Pharma, 2002). The assay was conducted in accordance with OECD Guideline 471 using the plate incorporation method (first assay) and treat and plate method (second assay).

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

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

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

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.0 to 3600 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 3600 µg/ml were selected for the chromosome aberration assay. In the chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of

this investigation, xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

VII. Acid Fungal Protease 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 35.81 mg 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, acid fungal protease (AFP) from *T. reesei* was tested in five strains of *Salmonella tyhimurium* (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 AFP 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, AFP 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 AFP 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 AFP concentrate both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, AFP concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation up to and including the maximum recommended dosage level of 5000 µg/ml.

D. Acute dermal irritation in rabbits

This study was conducted according to the method recommended in the OECD Guideline No. 404. In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application (Scantox, 2006).

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays. The mean score for skin edema and erythema was 0.0. AFP is classified as “not a skin irritant”.

E. Ocular irritation in rabbits

This study was conducted according to the method recommended in the OECD Guideline No. 405. In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded 1 and 24 hours later. The right eye served as control. After the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage at 48 and 72 hours after the treatment. A confirmatory test was conducted with 2 rabbits (Scantox, 2006).

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, no irritation was observed. The primary eye irritation score was 0.0. Under the conditions of this assay, AFP is classified as “not an eye irritant”.

F. 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 (Acute 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), AFP can be classified as non-hazardous (oral LD₅₀ > 2000 mg/kg bw).

Appendix 4

Appendix 4: Pariza GRAS Assessment

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

Michael W. Pariza, Member

June 19, 2007

Alice J. Caddow
Vice President, Regulatory
And Environmental Affairs
Genencor International, Inc.
925 Page Mill Road,
Palo Alto, CA 94304

Dear Ms. Caddow:

I have reviewed the information you provided on Genencor Danisco's Bovine chymosin B that is produced in *Trichoderma reesei* Pent CHY-Bip3, a recombinant strain derived from *T. reesei* RL-P37. In this evaluation I considered the biology of *T. reesei*, information available in the peer-reviewed scientific literature, and additional 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. *T. reesei* RL-P37, and enzyme preparations derived from this organism including cellulase, beta-glucanase, xylanase, acid fungal protease, alpha-amylase and glucoamylase, are well recognized as safe by qualified experts. Published literature, government laws and regulations for example FR 64:28658-28362; 1999, reviews by expert panels such as FAO/WHO JECFA (1992), well as Danisco's unpublished safety studies, support this conclusion. The new chymosin B production strain, *T. reesei* Pent CHY-Bip3, is derived from this safe strain lineage.

The *Bos taurus* chymosin B gene and its enzyme product (chymosin B) have a long history of safe use in cheese-making both in their original form (derived from calf

stomach) and from production microorganisms expressing cloned *Bos taurus* chymosin B genes. The methodology used to clone the *Bos taurus* chymosin B gene into *T. reesei* RL-P37 to produce the new production strain, *T. reesei* Pent CHY-Bip3, did not involve insertion of antibiotic resistance markers or any other DNA fragments that could result in the synthesis of products that might present a safety concern. Viable production organisms are not present in the final chymosin B enzyme product

The specifications you have developed for the chymosin B enzyme product are appropriate for a food grade enzyme. The product meets or exceeds standards set forth in Food Chemicals Codex and FAO/WHO JEFCA. I have also reviewed your recent production changes and agree with your assessment that they are appropriate for a food grade enzyme. Accordingly your new Genencor chymosin B enzyme product is substantially equivalent to chymosin enzyme products currently in commerce. Since the new Genencor chymosin B enzyme product will simply replace chymosin enzyme products currently in commerce, additional consumer exposure to chymosin is not anticipated as a result of this application.

Based on this information, I concur with the evaluation made by Genencor Danisco, that the new Genencor Danisco *T. reesei* Pent CHY-Bip3 production organism that expresses the *Bos taurus* chymosin B enzyme product is safe to use for the manufacture of food grade chymosin B. I further conclude that the chymosin B enzyme preparation manufactured using this organism by the process you described is GRAS for use as a food-processing ingredient.

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
Wisconsin Distinguished Professor of
Food Microbiology and Toxicology
Member, Michael W. Pariza Consulting LLC

Appendix 5

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

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the Chymostar Supreme product 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 3;
3. **Issues relating to the introduced DNA are addressed:**
 - a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** – Yes, rDNA derived chymosins have been accepted as safe and used to make cheese since the early 1990s. Go to 3c;
 - c. **Is the test article free of transferable antibiotic resistance gene DNA?** – Yes, go to 3e;
 - e. **Is all other introduced DNA well characterized and free of attributes that would render it un-safe for constructing microorganisms to be used to produce food-grade products?** – Yes, go to 4;
4. **Is the introduced DNA randomly integrated into the chromosome?** – Yes, inserted DNA is well characterized, but complete characterization of the location of all insertions is not possible 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 methods that were employed?** – In general, yes except as noted in 4. Go to 6;
6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** – Yes, *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing; Genencor has conducted toxicology testing on 7 different products derived from this lineage and all were determined to be safe for their intended food uses; Accept.

Conclusion: Article is accepted