A Protease Enzyme Preparation from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DUPONT AUSTRALIA PTY LTD
Submitted by: AXIOME PTY LTD

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EXECUTIVE SUMMARY

DuPont Industrial Bioscience (IB) is seeking approval for an Aspergillopepsin I enzyme product for use in processing of all food raw materials which naturally contain proteins. The enzyme is herein designated as Acid Fungal Protease (AFP).

AFP is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress a native *T. reesei* protease enzyme, Aspergillopepsin I.

AFP will replace other proteases currently marketed for the intended uses. AFP will be used in potable alcohol production and protein processing.

In all of these applications, AFP will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

To assess the safety of the AFP for use in potable alcohol production and protein processing, DuPont IB vigorously applied the criteria identified in the guidelines utilizing enzyme safety data, the safe history of use of other enzyme preparations from *T. reesei* and of other proteases in food, the safe history of use of the production organism for the production of other enzymes used in food, and a comprehensive survey of the scientific literature.

The safety of the food enzyme from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the DuPont *T. reesei* Safe Strain Lineage. The most suitable standard package of toxicological tests from the Safe Strain Lineage was identified to support the safety of the food enzyme object of the current dossier. The toxicological tests showed the following results:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL) is 1000 mg TOS/kg bw/day, which is the high dose in the study

Based on a conservative assumption and a highly exaggerated value consumption data, the NOAEL still offers a 340 fold Margin of Safety.

Based on the results of safety studies and other evidence, AFP has been demonstrated as safe for its intended applications and at the proposed usage levels. Approval of this application would provide manufacturers with benefits of facilitating the process and lower the manufacturing cost in potable alcohol production and protein processing.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Axiome Pty Ltd on behalf of Dupont Australia Pty Ltd

(b) Company:

DUPONT AUSTRALIA PTY LTD

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(Danisco Singapore Pte Ltd is a subsidiary of E. I. du Pont de Nemours and Company)

(e)

(f) Nature of Applicants Business:

DUPONT AUSTRALIA PTY LTD – A subsidiary of E. I. du Pont de Nemours and Company, manufacturer/marketer of specialty food ingredients, food additives and food processing aids.

Axiome Pty Ltd – regulatory & scientific affairs consultants

(g) Details of Other Individuals etc.:

No other individuals, companies or organizations are associated with this application.

1.2 Purpose of the application

This application seeks to modify Schedule 18 Section S18-4 Permitted Enzymes – Enzymes of Microbial Origin for Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application.

This application is made solely on behalf of DuPont Industrial Biosciences (IB), the manufacturer/marketer of the *Processing Aid*. When approved, the *Processing Aid* would be available for use by any food manufacturer in Australia and New Zealand.

Approval of this application would require amendment to Schedule 18 Section S18—4(5) Permitted Enzymes – Enzymes of Microbial Origin:

Table 1: regulatory impact statement.

Aspergillopepsin I (EC 3.4.23.18)	*Aspergillus niger* *Aspergillus oryzae* *Trichoderma reesei*

Currently no Aspergillopepsin I from *T. reesei* is permitted as a Processing Aid. However other enzymes including Cellulase, Endo-1,4–beta-xylanase, β-glucanase, Hemicellulase multicomponent enzyme, and Polygalacturonase or Pectinase multicomponent enzyme from *T. reesei* are listed in Schedule 18 Section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Section 2.3 and Appendix A.

1.3 Justification for the application

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application

AFP is an enzyme preparation produced by submerged fermentation of *T. reesei* carrying the gene encoding the acid fungal protease from *T. reesei.* The enzyme is characterized as an Aspergillopepsin I (EC 3.4.23.18). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme preparation for use in the applications outlined in Section 4.

AFP will replace other proteases currently marketed for the intended uses in potable alcohol production and protein processing.

More information on the benefit of this enzyme can be found in Section 2.2.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no Aspergillopepsin I from *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade

The inclusion of AFP from *T. reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product, and reduce technical barriers to trade.

1.4 Support for the application

No marketing or promotional activities have been undertaken for AFP containing the gene for Aspergillopepsin I from *T. reesei* in the Australia/New Zealand market for food. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5 Assessment procedure

This application seeks to modify Schedule 18 Section S18-4(5) for Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, DuPont IB considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6 Confidential Commercial Information

Certain (identified) technical and manufacturing information included in Appendices B1, B2, B3, B4, D4, D5 and Appendix E is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

1.7 Exclusive capturable commercial benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8 International and other National Standards

Refer to Appendix D for further details

1.8.1. Codex Standards

AFP produced by *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2. International Legislation

AFP has been determined to be GRAS in the United States as a food processing aid in grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of non-citrus juice (i.e., apple juice), the degumming of membranes during orange juice manufacture, and potentially other similar processes by a panel of scientific experts in the USA.

1.8. Statutory declaration

I,

of Australia, regulatory affairs consultant:

make the following declaration under the *Statutory Declarations Act 1959:*

1) The information provided in this application fully sets out the matters required

2) The information provided in this application is true to the best of my knowledge and belief

3) No information has been withheld which might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence Section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Signature: _____

Declared at _____ on _____ of _____

Before me,

Signature: _____

1.9. Checklist

CHECKLIST FOR STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD

This checklist will assist you in determining if you have met the information requirements as detailed in the Application Handbook. Section 3.1 – General Requirements is mandatory for all applications. Sections 3.3.1-3.3.3 are related to the specifics of your application and the information required is in addition to section 3.1.

General Requirements (3.1)

☒ Form of application	☒ Assessment procedure
☒ Applicant details	☒ Confidential Commercial Information
☒ Purpose of the application	☒ Exclusive Capturable Commercial Benefit
☒ Justification for the application	☒ International standards
☒ Information to support the application	☒ Statutory Declaration

Food Additives (3.3.1)

☐ Support for the application	☐ Analytical detection method
☐ Nature and technological function information	☐ Toxicokinetics and metabolism information
☐ Identification information	☐ Toxicity information
☐ Chemical and physical properties	☐ Safety assessments from international agencies
☐ Impurity profile	☐ List of foods likely to contain the food additive
☐ Manufacturing process	☐ Proposed levels in foods
☐ Specifications	☐ Percentage of food group to contain the food additive
☐ Food labelling	☐ Use in other countries (if applicable)

Processing Aids (3.3.2)

☒ Support for the application	☒ Information on enzyme use on other countries (enzyme only)
☒ Type of processing aid	☒ Toxicity information of enzyme (enzyme only)
☒ Identification information	☒ Information on source organism (enzyme from micro-organism only)
☒ Chemical and physical properties	☒ Pathogenicity and toxicity of source micro-organism (enzyme from micro-organism only)
☒ Manufacturing process	☒ Genetic stability of source organism (enzyme from micro-organism only)
☒ Specification information	☒ Nature of genetic modification (PA from GM micro-organism only)
☐ ~~Industrial use information (chemical only)~~	☒ List of foods likely to contain the processing aid

- ☐ Information on use in other countries (chemical only)
- ☐ Toxicokinetics and metabolism information (chemical only)
- ☐ Toxicity information (chemical only)
- ☐ Safety assessments from international agencies (chemical only)
- ☒ Anticipated residue levels in foods
- ☒ Percentage of food group to use processing aid
- ☒ Information on residues in foods in other countries (if available)

Nutritive Substances (3.3.3)

- ☐ Support for the application
- ☐ Identification information
- ☐ Information on chemical and physical properties
- ☐ Impurity profile information
- ☐ Manufacturing process information
- ☐ Specification information
- ☐ Analytical detection method
- ☐ Proposed food label
- ☐ Toxicokinetics and metabolism information
- ☐ Animal or human toxicity studies
- ☐ Safety assessments from international agencies
- ☐ List of food groups or foods likely to contain the nutritive substance
- ☐ Proposed maximum levels in food groups or foods
- ☐ Percentage of food group anticipated to contain nutritive substance
- ☐ Food consumption data for new foods
- ☐ Information on use in other countries
- ☐ Food consumption data for foods with changed consumption patterns
- ☐ Nutritional purpose
- ☐ Need for nutritive substance in food
- ☐ Demonstrated potential deficit or health benefit
- ☐ Consumer awareness and understanding
- ☐ Actual or potential behaviour of consumers
- ☐ Demonstration of no adverse affects to any population groups
- ☐ Impact on food industry
- ☐ Impact on trade

2. Technical information

Refer to Appendix A for further details

2.1. Type of processing aid

AFP is an enzyme preparation produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding a native *T. reesei* protease enzyme, Aspergillopepsin I.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code Section 1.3.3-6 Enzymes.

2.2. Identity

a) Chemical/Common Name:

The systematic name of the principle enzyme activity is Aspergillopepsin I. Other names used are Aspergillopepsin A, Aspergillopepsin F, Aspergillopepdase A, Awamorin. Protease B, Protease Type VIII, Proteinase B, Trypsinogen kinase.

- EC number: 3.4.23.18
- CAS number: 9025-49-4

Biological source: Aspergillopepsin I is an enzyme preparation produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding a native *T. reesei* protease enzyme, Aspergillopepsin I.

b) Marketing Name of the Processing Aid:

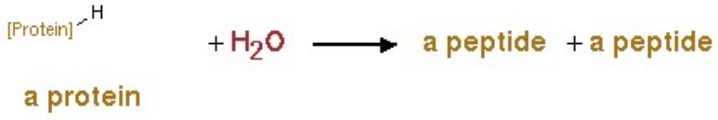
Fermgen™ 2.5X

c) Molecular and Structural Formula:

T. reesei AFP is a protein. The amino acid sequence is known. Refer to Appendix E.

2.3. Chemical and physical properties

AFP catalyses the following reaction. Hydrolysis of proteins with broad specificity. Generally favors hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. Does not clot milk. It can use proteins and peptides as a substrate.



AFP will be used in certain food processes including:

Potable alcohol production:

- "Predigestion" of the cereal proteins for optimal development of the fermentation

- Amylolytic enzymes have better access for the hydrolysis of the cereal starch granules

Protein Processing:

- Facilitate the production of peptides with better functional properties such as solubility (Cheng and Medina, 2012; Hasegawa et al., 1988), emulsification, gelling and foaming (Whitehurst and Law, 2010; Uhlig, 1998)

Description: The commercial enzyme preparation is a brown liquid.

Substrate specificity:

Aspergillopepsin I is one of several proteases produced by *T. reesei*. It causes hydrolysis of proteins with broad specificity. It generally favours hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. It does not clot milk.

Activity: The activity of the AFP is defined in SAPU/g (Spectrophotometric Acid Protease Unit). 1 SAPU/g is the activity which liberate 1 micromole of tyrosine equivalent per minute per gram of enzyme product under the conditions of the method.

Temperature optimum:

Approximately 50°C, with relatively high relative activity 40-65 °C.

Thermal stability: The enzyme activity rapidly decreases for temperatures above 55 °C . The enzyme is completely deactivated after incubating at 70°C for 15 min

pH optimum: approximately pH 3.2-4.2

pH stability: Optimal stability is seen at the pH interval 3.2 to 4.8 and the enzyme is relatively stable in the pH range 3-5.8.

Interaction of the enzyme with different foods:

The AFP enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Nutritional implication

AFP is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of AFP are very low, and as with other enzymes that are currently approved and used as Processing Aids use of this product would not have any nutritional significance.

2.4. Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of AFP is monitored and controlled by analytical and quality assurance procedures that ensure that the finished product complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5. Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of AFP to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 5.10^{+4} CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25g
Salmonella	absent in 25g
Antibiotic activity	negative by test
Production strain	absent
Physical properties:	
Appearance	liquid, brown

Standard for identity:

AFP meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

Allergenicity of the enzyme:

An allergen statement is given in Appendix A. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

3. Safety

Refer to Appendix B for further details

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e. to catalyze a specific chemical reaction. That reaction determines the IUBMB classification. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g. temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 1 below shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyze the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

Figure 1. Variation of enzymes in nature.

The expressed mature enzyme amino acid sequence of AFP shows clear conserved 'Aspergillopepsin_like' sequence domain, characteristic for aspartic proteases (IUBMB 3.4.23.18) of fungal origin. Our aspergillopepsin shows 51% identity to the aspergillopepsin from *A. oryzae*, which is one of the approved aspergillopepsin enzymes on Schedule 18 of the ANZ Food Standards Code. The identity between the ANFZ approved aspergillopepsins, from A. niger and A. oryzae, is 68%. Note that even available aspergillopepsin sequences obtained from different strains of one species show variability. For instance, an alignment of just three of the available *A. oryzae* aspergillopepsin amino acid sequences showed that these were 58-69% identical.

AFP derived from *T. reesei* carrying the gene encoding the native *T. reesei* Aspergillopepsin I enzyme, AFP has been determined to be GRAS in the United States,and approved by Denmark and France. AFP was introduced to market in 2003 in the U.S. and has been used in Canada, Brazil, Argentina, Europe, Thailand and other countries. The major use of this product in these countries are for potable alcohol production, with some uses in protein processing. There have not been any adverse events reported since AFP has been in commercial use in these countries.

Please refer to Section 1.8 and Appendix D for details on the different approval procedures in the countries listed above.

3.2. Toxicity of the enzyme

DuPont IB has determined by scientific procedures that production organism *T. reesei* NSP24 is safe as a production organism as it pertains to the DuPont *T. reesei* Safe Strain Lineage (see Appendix B).

Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) and is utilized by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the Total Organic Solid (TOS) originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Toxicological testing

Specific toxicology studies have not been performed with AFP from *T. reesei* expressed in the genetically modified strain of *T. reesei* NSP24. Instead, the safety of AFP from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the DuPont *T. reesei* Safe Strain Lineage. A review of toxicology studies conducted with enzyme preparations produced by *T. reesi* strains indicates that, regardless of the *T. reesei* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains, it is expected that any new enzyme preparation produced from *T. reesei* strains would have a similar toxicological profile.

DuPont IB has determined by scientific procedures that production organism *T. reesei* NSP24 is safe as a production organism as it pertains to the DuPont *T. reesei* Safe Strain Lineage (see Appendix B) – more specifically the '*T. reesei* Host Strain #4 (M1-1.1)' branch. The position of the food enzyme object of the current dossier as well as the position of the strain providing the supportive toxicological studies is presented in the DuPont *T. reesei* Safe Strain Lineage (Appendix B).

For the determination of the safety of AFP, we use the results of toxicology studies conducted on enzyme preparations derived from *T. reesei* strain'*T. reesei* (heterol. rDNA) Xylanase I strain' (Strain number XXII as in the SSL in Appendix B2 and Appendix B3).

The toxicological Data Set

Of all the studies conducted on enzyme preparations from *T. reesei* '*T. reesei* Host Strain #4(M1-1.1)' derived strains, the 90-day oral (gavage) study on strains given below provide robust data to assess the Safe Strain Lineage for the *T. reesei* strain.

- *'T. reesei* (heterol. rDNA) Alpha-amylase Strain'
- *'T. reesei* (heterol. rDNA) Xylanase Strain'

Collectively, the data support the concept of Safe Strain Lineage for the DuPont *T. reesei* production strain. Therefore, toxicology data obtained from production organisms derived from *T. reesei* could be applied to AFP and the extrapolation of toxicology information is in line with the Safe Strain Lineage concept of Pariza and Johnson (2001).

For the safety assessment of AFP from *T. reesei* NSP24, the data based on *T. reesei 'T. reesei* (heterol. rDNA) Xylanase I strain' with a NOAEL of 1,000 mg TOS/kg bw/day is used as bridging data. The toxicology data from *T. reesei 'T. reesei* (heterol. rDNA) Xylanase I strain' (Strain number XXII as in the SSL in Appendix B2 and Appendix B3) is selected for the following reasons:

1. The 90-day oral (gavage) study was conducted according to OECD guideline 408 and in compliance with all current GLP regulations.
2. Genotoxicity studies (Bacterial reverse mutation assay and *in vitro* chromosomal aberration assay with human peripheral lymphocytes) are available for *T. reesei 'T. reesei* (heterol. rDNA) Xylanase I strain'. The data show no evidence of genotoxicity.

In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3. Information on the source micro-organism

The production organism of the AFP preparation, the subject of this submission, is *T. reesei* strain NSP24. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance Aspergillopepsin I production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a.

Trichoderma reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). 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 recognized as 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 also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001).

Full details of the gene and recombinant microorganism are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.4. Pathogenicity and toxicity of the source micro-organism

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.

A literature search was conducted on August 28, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. The full search output is on file at DuPont IB . A review of the literature search uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human 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.

Two authors reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo et al. 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing.

Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen et al. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for human 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 preparations that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases in the Pariza and Johnson paper (Pariza and Johnson 2001) and various strains have been approved as commercial enzyme preparations internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (FDA 1999), France, Australia/New Zealand, China (MOH 1996), and Japan.

The donor organism is the parent strain of the host strain, *T. reesei* QM6a, therefore the safety assessment is the same.

3.5. Genetic stability of the source organism

The parental strain of the production strain *Trichoderma reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont IB and its parental companies, because of the stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using QM6a and its derivatives. Furthermore, the production strain has

demonstrated to be 100% stable after more than 60 generations of fermentation for AFP production. Refer also section 3.6.

3.6. Method used in the genetic modification of the source organism

The production organism of the AFP preparation, the subject of this submission, is *T. reesei* strain NSP24. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance Aspergillopepsin I production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps,from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a.

The donor organism is the parent strain of the host strain, *T. reesei* QM6a. Full details of the genetic modifications are provided in Appendix E (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by Southern Blot analysis. Broth samples were taken prior and after prolonged fermentation process. Samples were then used for genomic DNA extraction. Genomic DNA were digested with appropriate enzymes and probed with protease gene. As expected, consistent band patterns were observed in samples prior and after fermentation, indicating the protease expression cassette has been stably maintained through generations during the fermentation process.

Full details of the genetic modifications are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4. Dietary exposure

Refer to Appendix C for further details.

4.1. List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), AFP will be used in:

- Potable alcohol (14.2. Alcoholic beverages (including alcoholic beverages that have had the alcohol reduced or removed))
- Animal and Vegetable protein products (8.5 Animal products and 12.6 Vegetable protein products)

4.2. Levels of residues in food

The food enzyme object of the dossier is typically used in the following food manufacturing processes:

- Potable alcohol production
- Protein processing

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

The calculations are performed on the basis of the maximum amounts of the food enzyme that could theoretically be carried-over to final foods and drinks. In the present case, the values found for protein processing were used to calculate the Total TMDI, which was found to be:

2.938 mg TOS/kg body weight/day.

It must be emphasized that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of (among others) the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the food enzyme, and apply the highest recommended level;
- For the calculation of the TMDI's in food as well as in beverage, only those foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.

4.3. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The product would be used as a processing aid in about:

- 50% of the tonnage of potable alcohol sold in Australia and New Zealand
- 35% of vegetable and animal proteins sold in Australia and New Zealand

4.4. Levels of residues in food in other countries

The use levels of the AFP preparation in other countries are the same for those applications presented in Section 4.2.

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January 11, 2018

APPENDIX A: Technical Information

Contents:

Appendices A

A1	IUBUB Number
A2	CAS Number
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A4	Specifications of The Commercial Product
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1 Identity

1.1 Acid Fungal Protease (AFP)

The systematic name of the principle enzyme activity of AFP is Aspergillopepsin I. Other names used are Aspergillopepsin A, Aspergillopepsin F, Aspergillopepdase A, Awamorin. Protease B, Protease Type VIII, Proteinase B, Trypsinogen kinase.

AFP is an enzyme produced by submerged fermentation of *Trichoderma reesei* carrying the gene overexpressing a native *T. reesei* protease enzyme, Aspergillopepsin I.

- EC number: 3.4.23.18 (Appendix A1)
- CAS number: 9025-49-4 (Appendix A2)

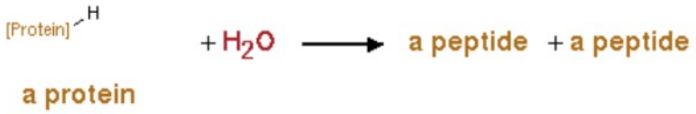
1.2 Other enzymes

Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and trace of other enzyme activities (e.g. other proteases) might be found but their level will be very low.

2 Chemical and physical properties

2.1 Substrate specificity

The food enzyme catalyses the following reaction:



Hydrolysis of proteins with broad specificity. Generally favours hydrophobic residues in P1 and P1′, but also accepts Lys in P1, which leads to activation of trypsinogen. Does not clot milk.

It can use proteins and peptides as a substrate.

2.2 Activity

The activity of the AFP is defined in SAPU/g (Spectrophotometric Acid Protease Unit). 1 SAPU/g is that activity which liberate 1 micromole of tyrosine equivalent per minute per gram of enzyme product under the conditions of the method.

This method is based on the release of solubilized casein peptides from a 30 minute proteolytic hydrolysis of a Purified High Nitrogen Casein Substrate at pH 3.0 and 37°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. Solubilized casein is then measured spectrophotometrically. One Spectrophotometric Acid Protease Unit (SAPU/g) is that activity which will liberate 1 micromole of tyrosine equivalents per minute per gram of enzyme product under the conditions of the method.

AFP has a minimum activity of 2500 SAPU/g. A detailed assay method is present in Appendix A3.

2.3 Temperature optimum

The activity of the food enzyme AFP from *T. reesei* was measured under temperature conditions using the analytical methods described below. Formulated AFP product was dosed volumetrically in all assays such that activity fell within the linear range of the activity assay,

below its saturation point (predetermined by performing a dose response experiment at pH 4.5, 50C). Activity was determined using a fluorescent assay where proteolytic activity on a conjugated bodipy-casein substrate is monitored by release of the bodipy dye (EnzChek® Protease Assay Kit, Life Technologies). Reaction incubations were performed in 100 ul volumes containing 10 ppm substrate with diluted enzyme in 50 mM potassium acetate buffer pH 4.5, 2 mM NaCl and 5 ppm calcium at 50C for 15 minutes in replicates of two in 96 well PCR tube format using PCR style heating blocks for incubation. End point fluorescence was measured using excitation and emission wavelengths of 485 and 530 nm, respectively, and is proportional to protease activity. The data presented is the fluorescence measured divided by enzyme concentration and reaction time. Fluorescence of the reactions at five minutes time intervals before quench also measure and found to correlate to the values measured at quench time. Curve was generated using GraFit 5.0.1 fitting to eq. (1):

$$Activity = {Activity_{Limit}}/{(10^{(pKa_1 - pH)} + 10^{(pH - pKa_2)} + 1)}$$

Temperature profiling was carried out in reactions at a 5000 fold dilution. All reactions were buffered with 50 mM potassium acetate pH 4.5 (with ions) and carried out over a 30-90°C range in increments of ~2°C using a gradient PCR block. The results are presented in Figure 1 below.

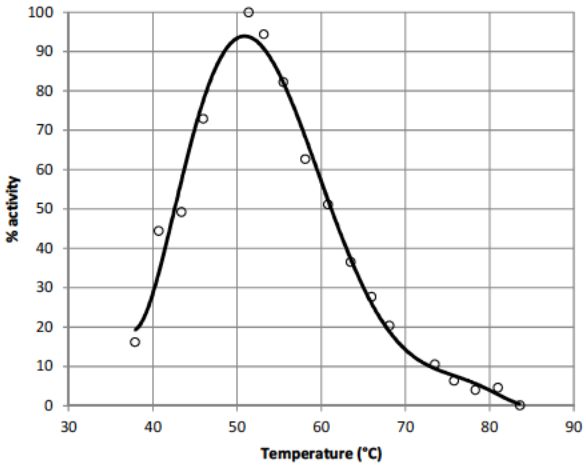


Figure 1. Effect of temperature on AFP activity

The curve shows that the AFP has temperature optimum around 50°C.

2.4 Thermal stability

To assess the stability of AFP, AFP enzymes were prepared at a 1000 fold dilution in pH 4.5 buffer as described in Section 2.3 except in replicates of three and pre-incubated at 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, and 100°C prior to assaying for activity as described in Section 2.3. Residual activities of pre-incubated enzymes were determined and were compared to that of enzyme that remained on ice prior to assay (control). Note that 100 ul activity assay reactions are assembled using 20 ul of diluted enzyme affecting a 5X dilution in assay reactions. The enzyme was present in the final assay reaction at a 5000 fold dilution.

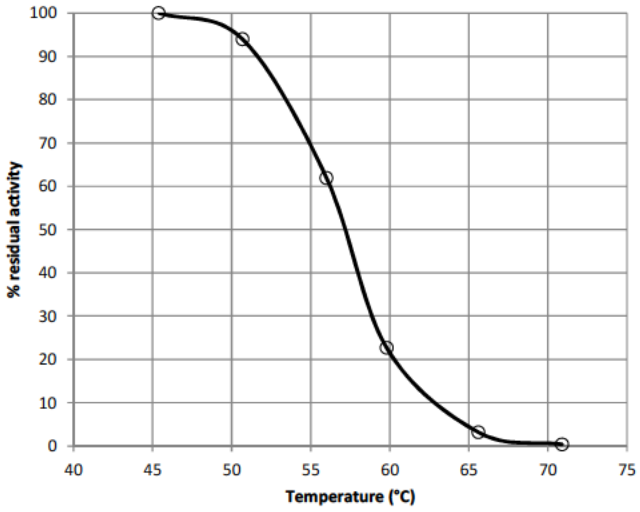


Figure 2. Thermal stability of AFP

The thermal stability of AFP is presented in Figure 2 above. The figure shows that the enzyme activity rapidly decreases for temperatures above 55 ºC. and is completely deactivated above 70 ºC.

2.5 pH optimum

The pH-activity profile was determined using a fluorescently labelled casein assay obtained from Molecular Probes (EnzChek Protease Kit –Green fluorescence) as described in section 2.3. Enzyme was diluted to 1.0 mg/ml, 0.5 mg/ml and 0.25 mg/ml. Fluorescently labelled substrate was diluted to 0.1mg/ml in ddH_2O. 10 ml of substrate was added to 50 ml of buffer of various pH and 30 ul of ddH_2O. Reactions were initiated by the addition of 10 ml of enzyme and allowed to continue for various amounts of time before being quenched by the addition of 100 ul of 1.0 M phosphate pH 10. End point fluorescence was measured using excitation and emission wavelengths of 485 and 530 nm, respectively.

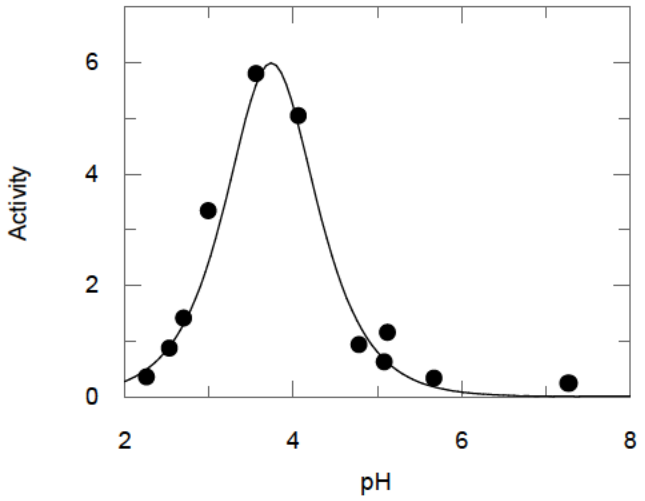


Figure 3. Effect of pH on AFP activity

The results (Figure 3) show that AFP has a pH optimum around pH 3.2 – 4.2.

2.6 Storage stability

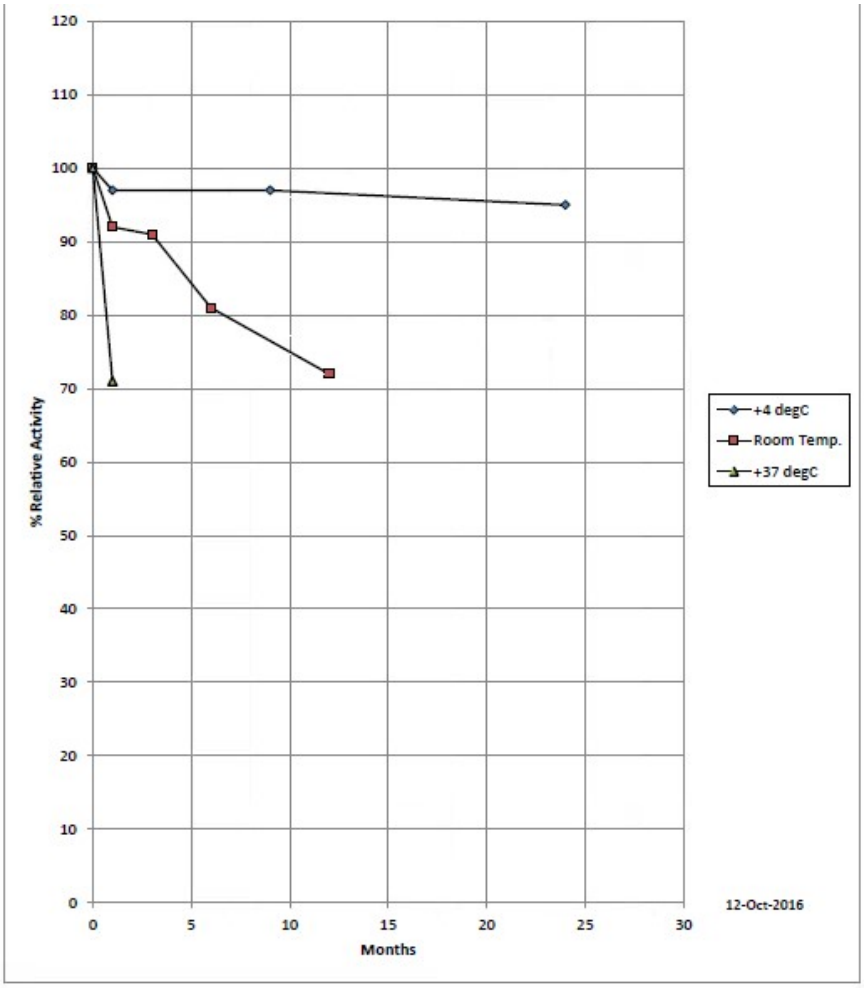


Figure 4: Stability of the AFP

At 4°C the enzyme is stable for more than 2 years without significant loss of activity.

3 Efficacy and benefits of the AFP enzyme preparation

3.1 Description

The benefits of the use of AFP in certain food processes may include:

Potable alcohol production:

- 'Predigestion' of the cereal proteins for optimal development of the fermentation
- Amylolytic enzymes have better access for the hydrolysis of the cereal starch granules

Protein processing:

- Facilitate the production of peptides with better functional properties such as solubility (Cheng and Medina, 2012; Hasegawa *et al.,* 1988), emulsification, gelling and foaming (Whitehurst and Law, 2010; Uhlig, 1998)

The above food processing has been extensively used for decades in the Australia and New Zealand and the rest of the world, which demonstrates the technological need of AFP in food production.

3.2 Efficacy examples

AFP is an acid proteolytic enzyme characterized by its ability to hydrolyze proteins under low pH conditions. The broad substrate specificity of AFP enables the enzyme to efficiently hydrolyze most grain proteins in a random fashion.

AFP provides the following benefits to ethanol producers (Figures 5-6):

- Higher yields and faster fermentation rates for corn, milo and wheat mashes. This is the result of starch freed from protein matrices and enhanced yeast nutrition by specific amino acids as well as di- and tripeptides.
- Reduces or eliminates the need for emulsion breakers in back end corn oil recovery systems.
- Reduces the amount of Nitrogen (Urea or Anhydrous Ammonia) needed in fermentation.
- Enhances yeast propagation and yeast performance.

Effect of AFP on Rate and Titer

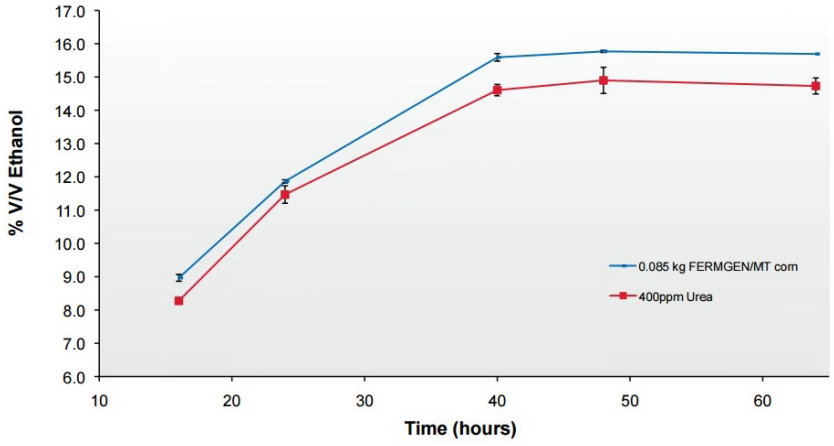
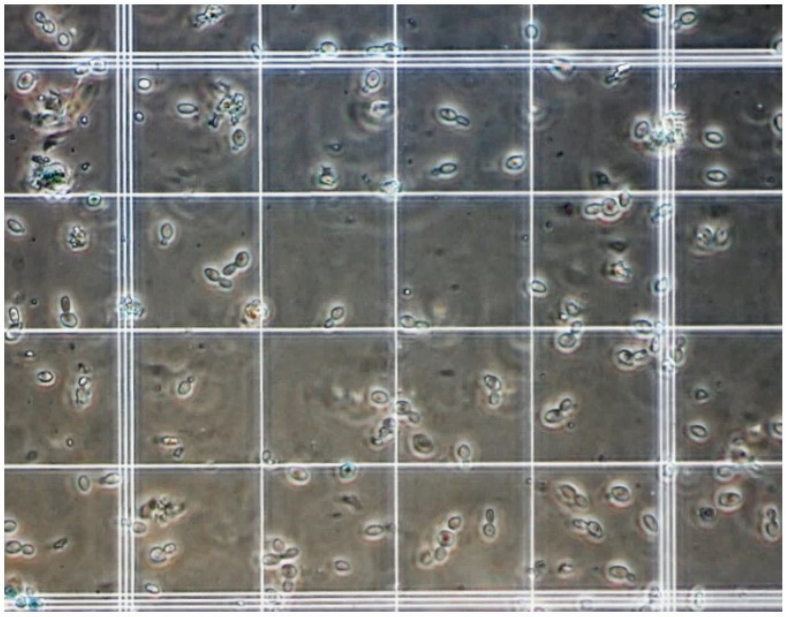
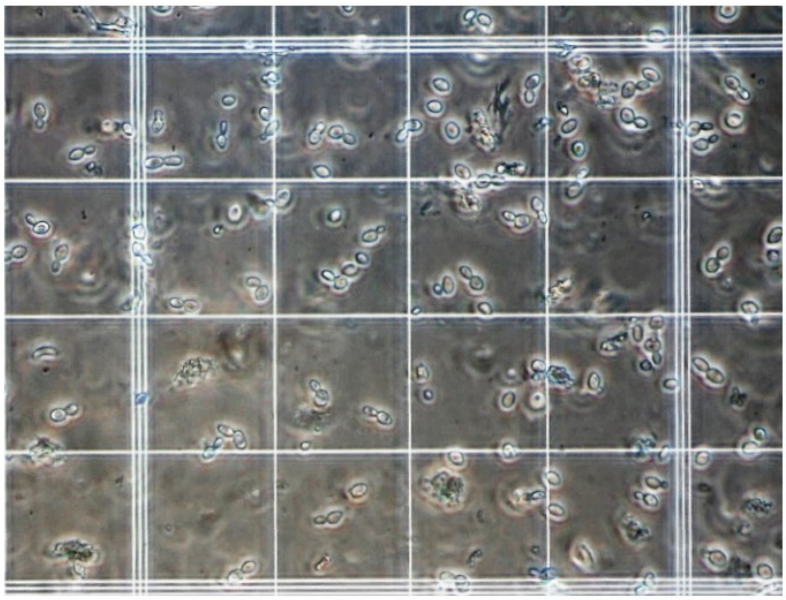


Figure 5: Effect of AFP on Rate and Titer

AFP significantly increases the rate of yeast propogation.



Control



AFP Dosage: 0.12 SAPU/g DS

Figure 6. Effect of AFP on yeast propagation

4 Manufacturing process

The manufacturing process for the production of AFP will be conducted in a manner similar to other food and feed production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for

enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation/ drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (see, Enzyme Applications, 1994; Aunstrup et al, 1979; Aunstrup, 1979).

4.1 Raw materials

The raw materials used in the fermentation and recovery process for the AFP enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008), 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. DuPont IB uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4.2 Fermentation

AFP is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* described in Appendix B. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. 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.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new lyophilized stock culture vial of the *T. reesei* production organism 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.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

4.3 Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e. AFP.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximize separation and is then fed into the filter or the centrifuge. The relatively solids free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g. glycerol to produce a liquid product.

A manufacturing flow sheet is found in Appendix A6.

4.4 Formulation

The ultrafiltrated concentrate is then formulated and analysed 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, 2006) and the FCC.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

5 Specification for identity and purity

5.1 Purity criteria

Appropriate GMP controls and processes are used in the manufacture of AFP to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits for the AFP product can be found in Appendix A4. Certificates of Analysis for three lots of product are given in Appendix A5.

The specifications for the AFP enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the Food Chemical Codex, 6th Edition (2008) (Appendix A7) and by the Joint FAO/WHO Expert Committee on Food additives (JECFA 2006) (Appendix A8).

5.2 Allergens

An allergen declaration of the enzyme concentrate can be found in Appendix A9.

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Appendix A1 : IUBUB Number

Source: IUBMB / **http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/23/18.html**

Accepted name: Aspergillopepsin I

Reaction: Hydrolysis of proteins with broad specificity. Generally favours hydrophobic residues in P1 and P1', but also accepts Lys in P1, which leads to activation of trypsinogen. Does not clot milk

Other names: *Aspergillus* acid protease; *Aspergillus* acid proteinase; *Aspergillus* aspartic proteinase; *Aspergillus awamori* acid proteinase; *Aspergillus* carboxyl proteinase; (see also Comments); carboxyl proteinase; *Aspergillus kawachii* aspartic proteinase; *Aspergillus saitoi* acid proteinase; pepsin-type aspartic proteinase; *Aspergillus niger* acid proteinase; sumizyme AP; proctase P; denapsin; denapsin XP 271; proctase

Comments: Found in a variety of *Aspergillus* species (imperfect fungi): *Aspergillus awamori* (awamorin, aspergillopepsin A: [8]), *A. foetidus* (aspergillopepsin F: [6]), *A. fumigatus* [7], *A. kawachii* [9], *A. niger* (proteinase B, proctase B: [2,4]), *A. oryzae* (trypsinogen kinase: [3,10]), *A. saitoi* (aspergillopeptidase A: [10]), and *A. sojae* [5,10]. In peptidase family A1 (pepsin A family). Formerly included in EC 3.4.23.6

Links to other databases: BRENDA, EXPASY, KEGG, MEROPS, Metacyc, PDB, CAS registry number: 9025-49-4

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[EC 3.4.23.18 created 1992 (EC 3.4.23.6 created 1961 as EC 3.4.4.17, transferred 1972 to EC 3.4.23.6, modified 1981 [EC 3.4.23.7, EC 3.4.23.8, EC 3.4.23.9, EC 3.4.23.10, EC 3.4.99.1, EC 3.4.99.15 and EC 3.4.99.25 all created 1972 and incorporated 1978], part incorporated 1992)]

Appendix A2 : CAS Number
Source: SciFinder Database

1. CAS Registry Number: 9025-49-4

CA Index Name: Aspergillopepsin I
Molecular Formula: Unspecified
References in CAplus: 676

Chemical Names

Other CA Index Names: Proteinase, Aspergillus acid

Synonyms: Amano 2G; Aspartic protease pepA; Aspergilloglutamic peptidase; Aspergillopepsin; Aspergillopepsin A; Aspergillopepsin B; Aspergillopepsin F; Aspergillopepsins; Aspergillopeptidase A; Aspergillopeptidases; Aspergillus acid protease; Aspergillus acid proteases; Aspergillus acid proteinase; Aspergillus acid proteinase; Aspergillus aspartic proteinase; Aspergillus aspartic proteinases; Aspergillus awamori acid protease; Aspergillus awamori acid proteinase; Aspergillus awamori acid proteinases; Aspergillus carboxyl proteinase; Aspergillus niger acid protease; Aspergillus niger acid proteinase; Aspergillus oryzae acid protease; Aspergillus saitoi acid protease; Aspergillus saitoi acid proteases; Aspergillus saitoi acid proteinase; Aspergillus-derived protease type-XIII; Avamorin; Avamorins; Awamorin; Bioprotease P conc.; Denapsin; Denapsin XP 271; E.C. 3.4.23.18; E.C. 3.4.23.6; E.C. 3.4.4.17; EC 3.4.23.18; EC 3.4.23.6; EC 3.4.4.17; Fungal acid protease; Fungal acid proteases; Genencor AFP 1000A; Molsin; Orientase 20A; Orientase 5A; Orientase AY; PepA; Proctase; Proctase B; Proctase P; Protease A; Protease A 2; Protease type-XIII; Proteinase M Amano; Proteinase M Amano G; Proteinase, Aspergillus awamori acid; Proteinase, Aspergillus kawachii aspartic; Proteinase, Aspergillus saitoi acid; Proteins, PepA; Sumizyme AP; Trypsinogen kinase; Type-XIII protease; Validase AFP

Other Identifiers

Deleted Registry Numbers: 169592-08-9; 39433-07-3; 37268-41-0; 102784-34-9; 9059-41-0

Appendix A3 : Activity of The Enzyme Complex

Determination of protease activity in SAPU (Spectrophotometric Acid Protease Unit) units

Substrate:

0.7% (w/v) Purified High Nitrogen Casein Substrate, pH 3.0

Assay procedure:

Pipet 10 mls of Casein Substrate into a series of 25 x 150 mm test tubes. Allow at least 2 tubes for each sample and 1 for each enzyme blank. Stopper the tubes and equilibrate them in a 37°C water bath for 15 minutes. Add 2 mls of enzyme dilution to each tube of equilibrated substrate, at timed intervals. Vortex and replace each tube in the water bath. Incubate tubes for exactly 30 minutes from the addition of the enzyme. At the same time interval used for the enzyme addition, add 10 mls of 1.8% TCA solution to stop the reaction in each tube. Vortex immediately after TCA addition. In the following order, prepare an enzyme blank containing 10 ml casein substrate, 10 ml TCA solution, and 2 mls of the appropriate enzyme dilution. Return all test tubes to the 37°C water bath for 30 minutes, allowing the precipitated protein to coagulate completely. After this incubation, transfer the tubes to an ice bath for 5 minutes. Filter each sample through Whatman No. 42 filter paper. The filtrate must be clear and free of any particles. Transfer all samples and blanks to plastic or quartz U.V. cuvettes and read the absorbance at 275 nm. Zero the spectrophotometer to distilled water.

Calculate the SAPU/g of enzyme product using the following equation:

$$\text{tyr equivalents/min. per g} = \text{SAPU/g} = \frac{(\text{tyr/ml})(22\text{ ml})}{(30\text{ min})(2\text{ ml sample})\left(\frac{\text{g enzyme}}{\text{ml buffer}}\right)}$$

Appendix A4: Specification of The Commercial Product

Analytical methods Certificate of Analysis

Analysis	Analytical method	JECFA specification
Total Coliforms	ISO 4832 - "Microbiology - General guidance for the enumeration of micro-organisms - colony count technique" and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Not more than 30 per gram
Escherichia coli	ISO 7521- "Microbiology - General guidance for the Enumeration Presumptive Escherichia coli - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Absent in 25 g of sample
Salmonella sp:	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No. 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Absent in 25 g of sample
Antimicrobial Activity	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Not detected
Lead	Mineral IPC QA-0245-2281 Based in # AOAC 984.2 - Calcium, Copper, Iron, Magnesium, Manganese, Phosphorus, Potassium, Sodium, and Zinc in Infant Formula Inductively Coupled Plasma Emission Spectroscopic Method.	Less than 5 mg/kg
Mycotoxin, T2	Neogen Corporation, Veratox T-2/HT-2 Toxins kit, V-T2HT2-0909	Negative
Production organism	SOP - Detection of production microorganism , R-SOP-SL-097-03	Absence

Appendix A5: Certificates of Analyses

CERTIFICATE OF ANALYSIS

PRODUCT: **AFP MVS Formulated Concentrate**

LOT NUMBER: **1682347169**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	SAPU/g	1000 - 3000	3613
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production Strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

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10-Nov-2014
Date

QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **AFP MVS Formulated Concentrate**

LOT NUMBER: **1682012977**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	SAPU/g	1000 - 3000	3098
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production Strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

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10-Nov-2014
Date

QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **AFP MVS Formulated Concentrate**

LOT NUMBER: **1682321162**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Protease	SAPU/g	1000 - 3000	3519
MICROBIOLOGICAL ANALYSIS			
Coliforms	CFU/ml	0 - 30	<10
E. coli	/25ml	Negative	Negative
Salmonella	/25ml	Negative	Negative
Production Strain	/ml	Negative	Negative
Antibacterial activity	/ml	Negative	Negative
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

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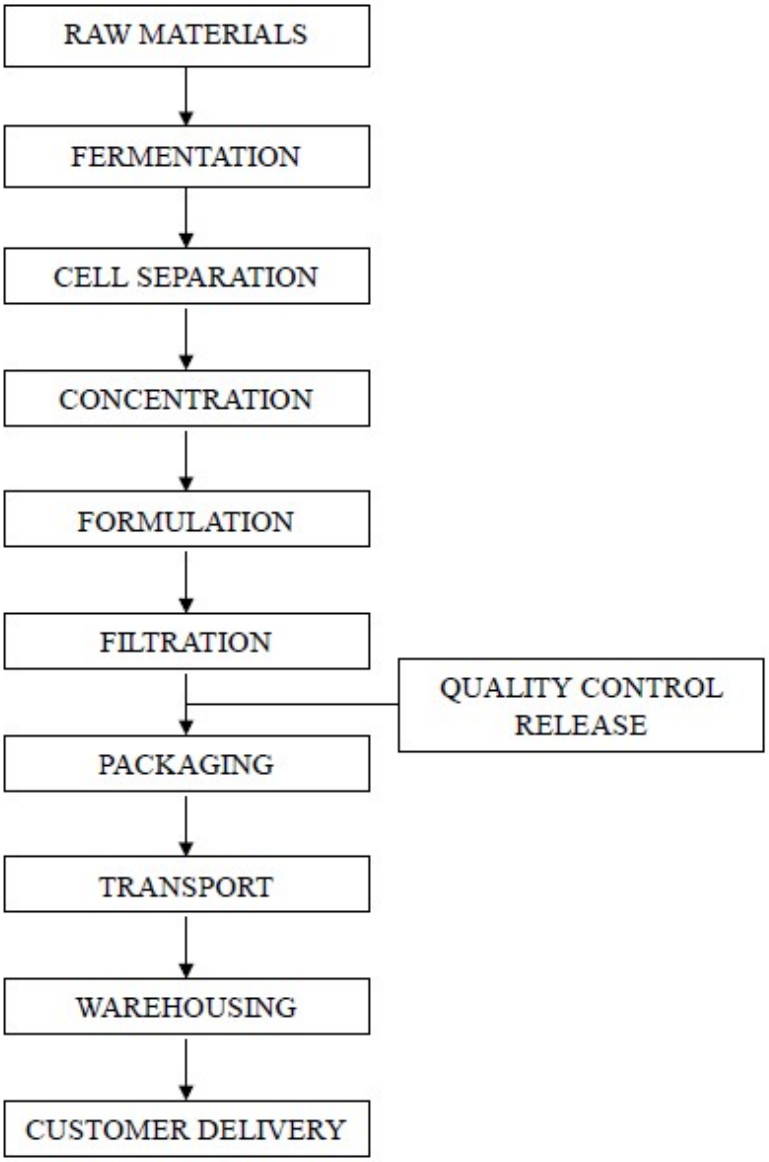
10-Nov-2014
Date

QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix A6: Production Process Flow Chart



Appendix A7: Food Chemical Codex, 6th edition

Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt, Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Enzyme Assays,* Appendix V.

Function Enzyme (see discussion under *Classification*, below)

Packaging and Storage Store in well-closed containers in a cool, dry place.

IDENTIFICATION

Classification

- **ANIMAL-DERIVED PREPARATIONS**

Catalase, Bovine Liver: Produced as partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from purified extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3)

lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueous extracts made from the fourth stomach of calves. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (chymosin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of lambs or kids.

Trypsin: Obtained from purified extracts of porcine or bovine pancreas. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: used in baking, in the tenderizing of meat, and in the production of protein hydrolysates.

- **PLANT-DERIVED PREPARATIONS**

Amylase: Obtained from extraction of ungerminated barley. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble

in water (the solution is usually colorless or light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

- **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase, pectin lyase*, and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosidase*, (6) *hemicellulase* (a mixture of enzymes, including *poly(galacturonate) hydrolase, arabinosidase, mannosidase, mannanase*, and *xylanase*), (7) *lactase*, (8) β-*glucanase*, (9) β-D-*glucosidase*, (10) *pentosanase*, and (11) α-*galactosidase*. Typical applications: used in the preparation of starch syrups and dextrose, alcohol, beer, ale, fruit juices, chocolate syrups, bakery products, liquid coffee, wine, dairy products, cereals, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortierella vinaceae* var. *raffinoseutilizer*) Produced as an off-white to tan powder or as pellets by controlled fermentation using *Mortierella vinaceae* var. *raffinoseutilizer*. Soluble in water (pellets may be insoluble in water), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*galactosidase*. Typical application: used in the production of sugar from sugar beets.

Carbohydrase: (*Rhizopus niveus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Rhizopus niveus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *glucoamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to

dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal, in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2)

catalase. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and cereal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase: (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*.

The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat cereals, and other grain-based foods.

- **REACTIONS CATALYZED**

[NOTE: The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Acetolactatedecarboxylase: Decarboxylation of α-cetolactate to acetoin

Aminopeptidase, Leucine: Hydrolysis of *N*-terminal amino acid, which is preferably leucine, but may be other amino acids, from proteins and oligopeptides, yielding free amino acids and oligopeptides of lower molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-**Amylase:** Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrow O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + O_2 ↔ D-glucono-δ-lactone + H_2O_2

β-D-**Glucosidase:** Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: Hydrolysis of α-1,4-glucan bonds

Lipase: Hydrolysis of triglycerides of simple fatty acids, yielding mono- and diglycerides, glycerol, and free fatty acids

Pancreatin:

α-Amylase: Hydrolysis of α-1,4-glucan bonds

Protease: Hydrolysis of proteins and polypepticles

Lipase: Hydrolysis of triglycerides of simple fatty acids

Pectinase:

Pectate lyase: Hydrolysis of pectate to oligosaccharides

Pectin depolymerase: Hydrolysis of 1,4 galacturonide bonds

Pectin lyase: Hydrolysis of oligosaccharides formed by pectate lyase

Pectinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase:

3-Phytase: *myo*-Inositol hexakisphosphate + H_2O ↔ 1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + H_2O ↔ an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α- and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

- **PROCEDURE**

Analysis: The following procedures, which are included under *Enzyme Assays,* Appendix V, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1]: Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

- **LEAD,** *Lead Limit Test,* Appendix IIIB

Control: 5 µg Pb (5 mL of *Diluted Standard Lead Solution*)

Acceptance criterion: NMT 5 mg/kg

SPECIFIC TESTS

- **MICROBIAL LIMITS**

[NOTE: Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.cfsan.fda.gov/.]

Acceptance criteria:

Coliforms: NMT 30 CFU/g

Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Change to read:

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source of derivation, they should cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used to produce enzymes must comply with the applicable U.S. meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used to produce enzymes or culture media used to grow microorganisms consist of components that leave no residues harmful to health in the finished food under normal conditions of use.

▲Preparations derived from microbial sources shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.▲ *FCC 6*

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

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[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
Monograph		(FI07) Food Ingredients Expert Committee

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Appendix A8 : General Specifications For Enzyme Preparations Used in Food Processing (JECFA)

General Specifications and Considerations for Enzyme Preparations Used in Food Processing

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (*1*) and published in FAO JECFA Monographs 1 (*2*). These specifications were originally prepared by the Committee at its twenty-fifth meeting (*3*) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (*4,5*). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (*6*). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 *(7*), and at the fifty-third meeting *(8*) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 *(9)*.

Classification and nomenclature of enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in processing and formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

% TOS = 100 - (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead:
Not more than 5 mg/kg.
Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:
Salmonella species: absent in 25 g of sample
Total coliforms: not more than 30 per gram
Escherichia coli: absent in 25 g of sample
Determine using procedures described in Volume 4.

Antimicrobial activity:
Absent in preparations from microbial sources.

Other considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and

the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.

2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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Appendix A9 : Allergen Declaration

Allergenic Ingredients

Product	FERMGEN 2.5X
Article-no.	A01734

The table below indicates the presence (as added component) of the following allergens and products thereof *. **Unless otherwise noted, the following listed allergens and products thereof have been used in the recovery process or in the formulation of an enzyme product:**

YES	NO	Allergens	Description of components
	(X)	Wheat	Glucose, (used in fermentation)**
	X	Other cereals containing gluten	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	X	Soybeans	
	X	Milk (including lactose)	
	X	Nuts (including but not limited to Almonds, Hazelnuts, Cashews, Brazilians, Macadamias, Pecans, Pistachios, Pinolis and Chestnuts)	
	X	Celery	
	X	Mustard	
	X	Sesame Seeds	
	X	Sulphur dioxide and sulfites >10mg/kg	
	X	Lupine and products thereof	
	X	Mollusk and products thereof	
	X	Natural Latex	

*Local legislation has always to be consulted as allergen labelling requirements may vary from country to country.

**DuPont IB has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements[1, 2].

January 11, 2018

APPENDIX B: Safety

Contents:

Appendices B

B1	Toxin homology search results (Confidential Commercial Information)
B2	Safe Strain Lineage (Confidential Commercial Information)
B3	Summary of safety studies on *Trichoderma reesei* derived enzymes in support of DuPont IB's Safe Strain Lineage (Confidential Commercial Information)
B4	Certificates of analyses of the test articles (Confidential Commercial Information)
B5	Risk assessment for potential food allergenicity
B6	Analysis of safety based on Pariza-Johnson Decision Tree

1 Toxicity of the enzyme

1.1 Toxin homology study

The *Trichoderma reesei* Protease (AFP) (mature) sequence is provided below (**Confidential Commercial Information**）:

Please refer to Appendix B Confidential Attachment.

The UniProt annotated Protein Knowledge database (Magrane et al., 2011; http://www.uniprot.org), release 2017_09 of 27-Sep-17, contains 555,594 reviewed proteins, of which 5826 sequences are manually annotated as toxins and 6275 as venom proteins (http://www.uniprot.org/biocuration_project/Toxins/statistics). These toxin and venom sequences are grouped in the animal toxin database subset (http://www.uniprot.org/program/Toxins).

A BLAST search for homology of the mature *T. reesei* protease (AFP) sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The vast majority of hits were proteases, with none of the top 1000 database hits being annotated as either toxin or venom.

A BLAST search for homology of the mature *T. reesei* protease (AFP) sequence was performed against the Uniprot animal toxin database. This yielded 2 hits, however, neither with an E-value lower than 0.1 and an identity higher than 35%. Therefore, the *T. reesei* protease sequence does not share homology with a known toxin or venom sequence.

Please refer to Appendix B1 Toxin Homology Search Results submitted separately as in the excel file for detailed analysis results (**Confidential Commercial Information**).

1.2 Toxicological testing

Specific toxicology studies have not been performed with AFP from *Trichoderma reesei* expressed in the genetically modified strain of *T. reesei* NSP24. Instead, the safety of AFP from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the DuPont IB *T. reesei* Safe Strain Lineage. A review of toxicology studies conducted with enzyme preparations produced by *T. reesi* strains indicates that, regardless of the *T. reesei* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ (see Appendix B2). Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains, it is expected that any new enzyme preparation produced from *T. reesei* strains would have a similar toxicological profile.

DuPont IB has determined by scientific procedures that production organism *T. reesei* NSP24 is safe as a production organism as it pertains to the DuPont IB *T. reesei* Safe Strain Lineage (see Appendix B1) – more specifically the '*T. reesei* Host Strain #4 (M1-1.1)' branch. The position of the food enzyme object of the current dossier as well as the position of the strain providing the supportive toxicological studies is presented in the DuPont IB *T. reesei* Safe Strain Lineage (Appendix B1).

For the determination of the safety of AFP, we use the results of toxicology studies conducted on enzyme preparations derived from *T. reesei* strain *'T. reesei* (heterol. rDNA) Xylanase strain'.

Of all the studies conducted on enzyme preparations from *T. reesei* '*T. reesei* Host Strain #4 (M1-1.1)' derived strains, the 90-day oral (gavage) study on strains given below provide robust data to assess the Safe Strain Lineage for the *T. reesei* strain.

- '*T. reesei* (heterol. rDNA) Alpha-amylase Strain'
- '*T. reesei* (heterol. rDNA) Xylanase Strain'

Collectively, the data support the concept of Safe Strain Lineage for the DuPont IB *T. reesei* production strain. Therefore, toxicology data obtained from production organisms derived from *T. reesei* could be applied to AFP and the extrapolation of toxicology information is in line with the Safe Strain Lineage concept of Pariza and Johnson (2001).

For the safety assessment of AFP from *T. reesei* NSP24, the data based on *T. reesei* '*T. reesei* (heterol. rDNA) Xylanase strain' with a NOAEL of 1000 mg TOS/kg bw/day is used as bridging data. The toxicology data from *T. reesei* '*T. reesei* (heterol. rDNA) Xylanase strain' is selected for the following reasons:

1. The 90-day oral (gavage) study was conducted according to OECD guideline 408 and in compliance with all current GLP regulations.
2. Genotoxicity studies (Bacterial reverse mutation assay and *in vitro* chromosomal aberration assay with human peripheral lymphocytes) are available for *T. reesei* '*T. reesei* (heterol. rDNA) Xylanase strain'. The data show no evidence of genotoxicity.

Assessment of genotoxicity

A. AMES TEST

A.1 Procedure

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

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

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

A.2 Results

In the screening assay, Xylanase X3 was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg TP/plate) in both the absence and presence of S-9 mix. No

positive mutagenic responses were observed with any of the tester strains in the presence of S-9 mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix. Based on the findings of the screening assay, 5000 µg TP/plate was selected as the highest dose level for the confirmatory assay.

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

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

A.3 Evaluation

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

B. CHROMOSOMAL ABERRATION TEST

B.1 Procedure

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

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

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

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

i. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays.

ii. Metaphase analysis (i.e., evaluation of chromosomal aberration) was conducted on at least 200 metaphases for each dose level (100 per duplicate treatment).

iii. Cells were scored for both chromatid-type and chromosome-type aberrations.

iv. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S9 and cultures with S9, respectively.

This assay was conducted in accordance with OECD guideline No. 473 (OECD, 1997b).

B.2 Results

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

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

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

B.3 Evaluation

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

C. Assessment of systemic toxicity

C.1. Test Performance

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

Animals of the same sex were housed in groups of two to three in solid floor polypropylene cages with stainless steel mesh lids and softwood bedding (non-aromatic) with access to water via an automatic system and feed *ad libitum*. For environmental enrichment, the

animals were provided a supply of wooden chew blocks and cardboard fun tunnels. All groups were housed under controlled temperature, humidity and lighting conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Urinalysis, clinical chemistry and hematology were conducted at study termination. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 12 for the control and all treated groups. All animals were sacrificed at the end of the 13-week study. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

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

C.2. Results

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

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

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

C.3. Conclusion

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

D. Data reporting

All individual safety studies and reporting of data have been performed according to the respective OECD guidelines.

The Xylanase X3 test material used for toxicological testing is sterile filtered, unformulated ultra filtered concentrate (UFC). The composition and specifications of the test material are given in the Table 1 below (See Appendix B3): As can be seen in the table below, the data are representative for the commercial batches. As argued above, the tox lot is representative for this strain through the SSL concept.

Table 1. Composition and Specifications of The Test Material (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment.

Review of the toxicological and exposure data and conclusions

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

- No mutagenic or clastogenic activity under the given test conditions was observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1,000 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). The Total TMDI of the food enzyme is 2.938 mg TOS/kg body weight/day. Consequently, the MoS is:

MOS = 1,000 mg TOS/kg body weight/day / 2.938 mg TOS/kg body weight/day

= 340

The Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

2 Information on the source micro-organism

The function of the genetic modification is to over express a native *T. reesei* protease enzyme, Aspergillopepsin I, using a known safe *T. reesei* host strain.

2.1 The production strain

Trichoderma reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). 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 recognized as 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 also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). In the subject microorganism, we have inserted into this strain copies of the gene encoding Aspergillopepsin I, isolated from the host strain *T. reesei*, that has been placed under the control of the high efficiency promoter obtained from the CBHI encoding gene. This strain was obtained by modification of earlier production strains, which are therefore included in the description as intermediate strains. The history of the strain development, method of transformation and the genetic tools used to transform the host strain are described in Appendix E.

2.2 The host

The host organism *T. reesei* strain RL-P37 was obtained from Dr. Montenecourt. The derivation and characterization of strain RL-P37 has been published (Sheir-Neiss and Montenecourt, 1984). Strain RL-P37 is a cellulase over-producing strain that was obtained through several

classical mutagenesis steps from the wild-type *T. reesei* strain (QM6a). Strain QM6a is present in several public culture collections, e.g. in the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina* (Kuhls *et al*., 1996; Dugan, 1998).

2.3 The donor organism

The donor strain is *T. reesei* QM6a, the parent of host strain RL-P37. The AFP gene for Aspergillopepsin I was isolated from strain QM6a.

2.4 The vector

Appendix E includes a full description of the scheme for the construction of the production strain. Appendix E2 provides a detailed description or the production strain and the steps employed to construct it. Appendix E3 provides the amino acid sequences of the AFP enzyme.

Only the intended acid fungal protease expression cassette has been integrated into the chromosomal DNA of *T. reesei* host strain (Please see Appendix E for details). No bacterial vector DNA remained present in the final production strain.

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.

3 Pathogenicity and toxicity of the modified micro-organism

3.1 The production strain

Trichoderma reesei was first isolated from nature in 1944. The original isolate, QM6a (Mandels and Reese, 1957), 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 *Trichoderma longibrachiatum* (Bissett 1984). Subsequent evidence pointed out that the two species are not identical (Meyer *et al*. 1992) even though several regulatory jurisdictions still use both names interchangeably. The proposal by Khuls *et al*. (1996) that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *Hypocrea jecorina* and no longer includes it in the genus Trichoderma. Therefore, *T. reesei*, *T. longibrachiatum,* and *Hypocrea jecorina* may appear in different documents and national positive lists, but for historical reasons they refer to essentially the same microorganism species.

A literature search was conducted on August 28, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. The full search output is on file in DuPont IB. A review of the literature search uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals. The species is not present on the list of pathogens used by the EU (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.

Two authors reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo *et al.* 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing.

Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

Trichoderma reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer *et al.* (2006), and various strains have been approved for the manufacture of commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, Australia/New Zealand, China, and Japan.

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

3.2 The donor

Since the donor organism is also *T. reesei,* please refer to section 3.1. above.

3.3 The host

Trichoderma reesei is not listed in Annex III of Directive 2000/54/EC – which lists microorganisms for which safety concerns for workers exist, as it is globally regarded as a safe microorganism:

- In the USA, *T. reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements1, if this fungus was to be used in submerged standard industrial fermentation for enzyme production.
- In Europe, *T. reesei* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German

Federal Institute for Occupational Safety and Health (BAuA) and the Federal Office of Consumer Protection and Food Safety (BVL), and not appearing on the list of pathogens from Belgium.

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992).

Cellulase, glucanase and glucoamylas from *T. reesei* have been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set (Monograph (FNP 52 (1992), JECFA, Monograph 14 (2013, JECFA 77th).

Cellulase, Endo-1,4-beta-xylanase, β-glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* have been approved as processing aid by FSANZ.

Cellulase from *T. reesei* was affirmed as GRAS by U.S. FDA (21CFR184.1250). Also the FDA has no questions to four GRAS notices on enzymes derived from *T. reesei*:

- Pectin lyase derived from *T. reesei* carrying a gene encoding pectin lyase from *Aspergillus niger* (GRN 32)
- Transglucosidase enzyme preparation from *T. reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* (GRN 315)
- Chymosin enzyme preparation from *T. reesei* expressing the bovine prochymosin B gene (GRN230)
- Glucoamylase enzyme preparation from *T. reesei* expressing the glucoamylase gene from *T. reesei* (glucoamylase enzyme preparation) (GRN372)

T. reesei has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. During recent years, genetic engineering techniques have been used to improve the industrial production strains of *T. reesei* and considerable experience on the safe use of recombinant *T. reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *T. reesei.* Thus, *T. reesei* can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for other safe gene products.

3.4 Allergenicity of AFP

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 function. 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. Like many other proteins, enzymes may have the potential to cause allergic responses, primarily after inhalation exposure. According to Pariza and Foster (1983) allergies represent only a very minor food safety concern in

regard to food processing enzymes. Allergic reactions after consuming enzymes could happen, but are scarce (Dauvrin et al, 1998). Poulsen (2004) reported that ingestion of an enzyme does not commonly result in the corresponding food allergy in individuals with inhalation allergy to a particular enzyme. Bindslev-Jensen et al (2006) conducted extensive studies in individuals with allergies with a variety of ingested food enzymes (carbohydrases, lipases, proteases) and confirmed that they are not food allergens, regardless of microbial source (bacterial or fungal) or the techniques used to produce them, including rDNA modification and protein engineering. These and other reports allow us to conclude that ingestion of food enzymes is not considered to be a concern with regard to food allergy. This may due to difference in exposure pattern (digestive route vs. inhalation route), insignificant exposure level in final foods, inactivation through processing, or molecular structure.

Despite this lack of general concern, the potential that AFP could be a food allergen was examined (for details, see Appendix B4).

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics et al. (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics et al. (2011) further discussed the use of the "E- score or E- value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." The search for 80 - amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database [1] (http://www.allergenonline.org/index.shtml) containing 2035 peer - reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) released on January 18, 2017[2] revealed multiple stretches throughout the peptide sequence with over 35% identity to:

- Aspergillopepsin I, an aspartic proteinase, EC 3.4.23.2.18, from *A. fumigatus* (NCBI gi|963013), also referred to as *Asp f* 10. The maximum sequence identity to the allergen was 63.79% with an E-score[3] of $1.4x10^{-70}$ and;
- Pepsin A, a protease, EC 3.4.23.1, from precursor *Sus scrofa* (NCBI gi|118572685). The maximum sequence identity to the allergen was 42.51% with an E-score of $4.3x10^{-20}$ and;
- Endopeptidase, EC 3.4.23 (NCBI gi|695094784) from *Rhizopus oryzae* with an identity of 40.50%, and an E-score of $1.1x10^{-20}$.

Asp f 10 is not identified as a food allergen by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee[4] .

[1] http://www.allergenonline.org/index.shtml
[2] http://www.allergenonline.org/databasebrowse.shtml
[3] The AllergenOnline database help page (http://www.allergenonline.org/databasehelp.shtml) states:
"For a database the size of AllergenOnline, two sequences might be considered related in evolutionary terms (i.e. diverged from a common ancestor and share common three-dimensional structure), when the E-value of the FASTA query is less than 0.02 (Pearson, 1996). However, a value of 0.02 does not mean that the overall structures are likely to be sufficiently similar for antibodies (*e.g.* IgE from an allergic individual) against one protein to recognize the other. To identify proteins that may share immunologic or allergic cross-reactivity, matches with E-values larger than 10^{-7} are not likely to identify relevant matches, while matches with E-values smaller than 10^{-30} are much more likely to be cross-reactive in at least some allergic individuals (Hileman, 2002). Since E-values depend to a great degree on the scoring matrix, the size of the database and many other factors, interpretation of immunological significance should be viewed with caution. As such, it is recommended to use a conservative E score value (e.g. 10^{-7}) as an additional data point to complement the percent identity score.
[4] http://www.allergen.org/viewallergen.php?aid=98

FASTA alignment of the sequences described above with known allergens also using the AllergenOnline database (http://www.allergenonline.org/index.shtml) revealed no match (using E-value <0.1 as the cut -off) to sequences in the data base using the full sequence search capabilities. Performing the same searches with the same criteria using the Allermatch database (http://www.allermatch.org/; Fiers et al.,2004) did not reveal any hits either. Neither the search for 80 amino acid stretches within the sequence with greater than 35% identity to known allergens, the full FASTA alignments (using E-value <0.1 as the cut -off), nor exact match of short contiguous search (8mer search) revealed any match.

In conclusion, based on the sequence homology alone, AFP is unlikely to pose a risk of food allergenicity.

4 Genetic stability of the source organism

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

5 Pariza-Johnson Decision tree

Pariza and Johnson (2001) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations.

DuPont IB has evaluated AFP according to the safety scheme of Pariza and Johnson (2001) (Appendix B5) and determined that this enzyme preparation is safe for use in food as a processing aid. This determination employed an extensive review of published and unpublished safety data available on the enzyme, the production organism, the enzyme manufacturing process, and the enzyme product (Pariza and Johnson, 2001).

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Appendix B1: Toxin homology search results (Confidential Commercial Information)

Please refer to Excel file submitted separately (**Confidential Commercial Information)**

Appendix B2: Safe Strain Lineage (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment.

Appendix B3: Summary of safety studies on *Trichoderma reesei* derived enzymes in support of DuPont IB's Safe Strain Lineage (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment.

Appendix B4: Certificates of analyses of the test articles (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment.

Appendix B5: Risk assessment for potential food allergenicity

Sequence Analysis Based Risk Assessment for Potential Food Allergenicity of *Trichoderma reesei* Protease (AFP) expressed in *Trichoderma reesei.*

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

Trichoderma reesei Protease (AFP) (mature) sequence is given below in FASTA format (Confidential Commercial Information).

Please refer to Appendix B Confidential Attachment.

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[5] containing 2035 peer-reviewed allergen sequences released on January 18, 2017[6] (Supplementary 1), revealed multiple stretches throughout the peptide sequence with over 35% identity to:

- Aspergillopepsin I, an aspartic proteinase, EC 3.4.23.2.18, from *A. fumigatus* (NCBI gi|963013), also referred to as *Asp f* 10. The maximum sequence identity to the allergen was 63.79% with an E-score[7] of $1.4x10^{-70}$ and;
- Pepsin A, a protease, EC 3.4.23.1, from precursor *Sus scrofa* (NCBI gi|118572685). The maximum sequence identity to the allergen was 42.51% with an E-score of $4.3x10^{-20}$ and;
- Endopeptidase, EC 3.4.23 (NCBI gi|695094784) from *Rhizopus oryzae* with an identity of 40.50%, and an E-score of $1.1x10^{-20}$.

[5] http://www.allergenonline.org/index.shtml
[6] http://www.allergenonline.org/databasebrowse.shtml
[7] The AllergenOnline database help page (http://www.allergenonline.org/databasehelp.shtml) states:
"For a database the size of AllergenOnline, two sequences might be considered related in evolutionary terms (i.e. diverged from a common ancestor and share common three-dimensional structure), when the E-value of the FASTA query is less than 0.02 (Pearson, 1996). However, a value of 0.02 does not mean that the overall structures are likely to be sufficiently similar for antibodies (*e.g.* IgE from an allergic individual) against one protein to recognize the other. To identify proteins that may share immunologic or allergic cross-reactivity, matches with E-values larger than 10^{-7} are not likely to identify relevant matches, while matches with E-values smaller than 10^{-30} are much more likely to be cross-reactive in at least some allergic individuals (Hileman, 2002). Since E-values depend to a great degree on the scoring matrix, the size of the database and many other factors, interpretation of immunological significance should be viewed with caution. As such, it is recommended to use a conservative E score value (e.g. 10^{-7}) as an additional data point to complement the percent identity score.

Asp f 10 is is not identified as a food allergen by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee[8].

FASTA alignment of the above sequence with known allergens also using the AllergenOnline database[1] did not reveal any matches (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities (Supplementary 1).

Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al*. (2011) and on AllergenOnline.org there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). This database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous amino acids search produced no sequence matches with known allergens (Supplementary 1).

Microbial enzymes acting environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a concern with regard to food allergy.

In conclusion, based on the sequence homology alone, *Trichoderma reesei* Protease (AFP) is unlikely to pose a risk of food allergenicity.

As for all enzyme products, an SDS for the Protease (AFP) product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

Reviewed and evaluated by:

References

Bindslev-Jensen, C, P Stahl Skov, EL Roggen, P Hvass, and D Sidelmann Brinch. 2006. Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem. Toxicol.* 44: 1909–1915

Codex Alimentarius Commission. 2009. Foods Derived from Modern Biotechnology, Annex 1, Assessment of Possible Allergenicity, Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Rome, Italy, http://www.fao.org/docrep/011/a1554e/a1554e00.htm, pp. 21-23

[8] http://www.allergen.org/viewallergen.php?aid=98

Herman, RA, Ping Song, and A ThirumalaiswamySekhar. 2009. Value of eight-amino-acid matches in predicting the allergenicity status of proteins: an empirical bioinformatic investigation. *Clinical and Molecular Allergy* 7: 9

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

Poulsen LK(2004). Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods. Mol. Nutr. Food Res. 48, 413 – 423

Appendix B5 Supplement 1 (Confidential Commercial Information).

Please refer to Appendix B Confidential Attachment

Appendix B6: 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 Food Pro LysoMax Oil 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. Acid Fungal Protease from *T. reesei* has always been a component of the enzyme mixture sold as cellulase and DuPont IB has sold it as a product produced by a different recombinantly modified strain since 2006. 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? –** 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 pleitropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? –** Yes, Go to 6;

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? –** Yes, Yes. The *T. reesei* host and methods of integration of the enzyme gene have been used by DuPont IB for production of many enzyme production organisms. Toxicology studies on 24 products from this strain lineage, including on this enzyme produced by an earlier recombinantly derived strain, confirms the safety of the lineage. **Accept.**

Conclusion: Article is accepted

January 11, 2018

APPENDIX C: Dietary Exposure

Contents:

1 Application areas

In below applications, AFP will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

The food enzyme object of the dossier is typically used in the following food manufacturing processes:

- Potable alcohol production
- Protein processing

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), AFP will be used in:

- Potable alcohol (14.2. Alcoholic beverages (including alcoholic beverages that have had the alcohol reduced or removed))
- Animal and Vegetable protein products (8.5 Animal products and 12.6 Vegetable protein products)

2 Level of use

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and AFP is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

The Table below shows the range of recommended use levels for each application where the food enzyme may be used.

Application	**Raw material (RM)**	**Recommended use levels (mg TOS/kg RM)**	**Maximal recommended use levels (mg TOS/kg RM)**
Potable alcohol production	Grist	5-50	50
Protein processing	Protein (e.g. vegetable, animal, microbial and milk proteins e.g. whey and casein)	30-1000	1000

3 Level of residues in food

3.1 Estimated Food Intake

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

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the assumed consumption of important foodstuffs and beverages as presented below. An additional assumption for foodstuff other than those considered to be important in the daily diet (e.g. dairy, meat, fish, poultry, vegetable or cereal products) to max constitute 50 % of the intake is added as is an additional assumption for Beverages that only 50 % of the 'Soft drinks' are prepared by use of the enzyme. The overall exposure becomes:

Average consumption over the course of a lifetime/kg body weight/day	**Total solid food (kg)**	**Total non-milk beverages (l)**	**Processed food (50% of total solid food) (kg)**	**Soft drinks (25% of total beverages) (l)**
	0.025	0.1	0.0125	0.025
			Non-essential food max constitute 50% of intake→ 0.00625	Only 50% of beverages are prepare by the enzyme→ 0.0125

* foodstuff other than those considered to be important in the daily diet (e.g. dairy, meat, fish, poultry, vegetable or cereal products)

3.2 Estimated intake of AFP

In Section 2 above, the recommended use levels of the enzyme AFP are given, based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material and it is assumed that all the TOS will end up in the final product. In the case of alcohol distillation, however, it is assumed that nothing of the TOS will end up in the final product due to the distillation process. Therefore, this application is not mentioned in the Table below.

Application		**Raw material (RM)**	**Maximal recommended use level (mg TOS/kg RM)**	**Example Final food (FF)**	**Ratio RM/FF**	**Maximal level in FF (mg TOS/kg food)**
Beverages	Protein processing	Protein (e.g. vegetable, animal, microbial and milk proteins e.g. whey and casein)	1000	Protein hydrolysates used in e.g. Sport drinks and shakes	0.15	150

Solid food	Protein processing	Protein (e.g. vegetable, animal, microbial and milk proteins e.g. whey and casein)	1000	Protein hydrolysates used in e.g. soups, bouillons, dressings, Sport and medical, Protein bars, Powders, Shakes.	0.17	170

The Total TMDI can be calculated on basis of the **maximal** values found in food and beverage (in the above cases, protein processing of liquid and solid food) multiplied by the average consumption of food and beverage/kg body weight/day.

Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
170x0.00625=1.0625	150x0.0125=1.875	**2.938**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme AFP from *T. reesei*.
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process, (except for alcohol distillation where it can safely be assumed that nothing of the TOS will end up in the final product. Therefore, this use was excluded from the calculation);
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass *et al*., 1997).

4 Safety assessment

Acid Fungal Protease (AFP) is derived from a selected non-pathogenic, non-toxigenic strain of *T. reesei* which is genetically modified to over express a native *T. reesei* protease enzyme, Aspergillopepsin I.

DuPont IB has determined by scientific procedures that production organism *T. reesei* NSP24 is safe as a production organism as it pertains to the DuPont IB *T. reesei* Safe Strain Lineage (see Appendix B1, B2) – more specifically the '*T. reesei* Host Strain #4' branch. For the determination of the safety of AFP, we use the results of toxicology studies conducted on enzyme preparations derived from *T. reesei* strain *'T. reesei* (heterol. rDNA) Xylanase I strain' (Strain number XXII as shown in the SSL and toxicological study summaries).

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

- No mutagenic or clastogenic activity under the given test conditions was observed;

- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1,000 mg TOS/kg body weight/day.

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

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

  $$\text{Margin of safety} = \frac{1{,}000 \text{ mg/kg bw/day}}{2.938 \text{ mg/kg bw/day}} = 340$$

5 Conclusion

The safety of AFP as a food processing aid in potable alcohol and protein hydrolysates is assessed with the Safe Strain Lineage concept and toxicology studies conducted on earlier strains of this lineage. Similar to all enzymes produced by the same DuPont IB *T. reesei* lineage, AFP produced by strain *T. reesei* NSP24 derived from strain 'T. reesei Host Strain #4' is not expected to be a mutagen, a clastogen, or an aneugen.

Based on a worst-case scenario that a person is consuming potable alcohol and protein hydrolysates treated with AFP (i.e., cumulative risk), this NOAEL still offers at least a 340X fold margin of safety.

6 References

Douglass JS, Barraj LM, Tennant DR, Long WR and Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

Hansen SC (1966). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet. Toxicol, 4, 427–432

MPI Research Inc., Study H-30536: Subchronic Toxicity 90-day oral gavage study in rats. Nov, 2013

January 11, 2018

APPENDIX D: International and Other National Standards

Contents:

Appendices D

D1	Dr Pariza GRAS letter (Mar, 2015)
D2	Dr Pariza GRAS letter (Sep, 2015)
D3	GRAS Notice 000333
D4	France Authorization of Aspergillopepsin I from *T.reesei* (Confidential Commercial Information)
D5	Denmark Authorization of Aspergillopepsin I from *T.reesei* (Confidential Commercial Information)
D6	EU Legislation on Enzymes

1 Codex Standards

1.1 The enzyme

Acid Fungal Protease from a recombinant strain of *Trichoderma reesei* has not been evaluated by the Joint Expert Committee on Food Additives (JECFA).

1.2 Supporting evaluations

Cellulase from *T. reesei* and Glucoamylase from *T. reesei* expressed in *T. reesei* have been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set (Technical Report Series 733, 1986).

2 International Legislation

2.1 United States

2.1.1 The enzyme

AFP has been determined to be GRAS as a food processing aid in grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of non-citrus juice (i.e., apple juice), and the degumming of membranes during orange juice manufacturing and potentially other similar processes, and in duel ethanol manufacture woth resulting distillers grains subsequently used in animal feed by an expert panel in Mar 2015 (See Appendix D1). In Sep 2015, the external panel was reconvened to review the additional use of the enzyme in fuel ethanol manufacture from grains and cane/molasses with resulting co-products (distillers' grains, corn gluten feed/meal, and vinasse) destined for use in animal feed (See Appendix D2).

The subject of GRN000333 is the same native *T. reesei* AFP enzyme preparation as the subject of this application, but produced by a different *T. reesei* strain within the safe linage (Appendix D3).

2.1.2 Supporting approvals

Cellulase from *Trichoderma reesei* were affirmed as GRAS by U.S. FDA (21CFR184.1250). Also the FDA has no questions to four GRAS notices on enzymes derived from *T. reesei*:

- Pectin lyase derived from *T. reesei* carrying a gene encoding pectin lyase from *Aspergillus niger* (GRN 32)
- Transglucosidase enzyme preparation from *T. reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* (GRN 315)
- Chymosin enzyme preparation from *T. reesei* expressing the bovine prochymosin B gene (GRN230)
- Glucoamylase enzyme preparation from *T. reesei* expressing the glucoamylase gene from *T. reesei* (glucoamylase enzyme preparation) (GRN372)

2.2 Europe

2.2.1 The enzyme

AFP has been approved in both France (Appendix D4) and in Denmark (Appendix D5).

In Europe, most of the enzyme preparations used in food processing are considered processing aids, meaning that they have their technological function in the food-processing stage and not in the final food. They are excluded from the Food Additives Framework Directive. On 16 December 2008 the European Parliament and the Council adopted

Regulation 1332/2008 EC on food enzymes which aims to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU (Appendix D4). Several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable.

Only France and Denmark have legislation covering all food-use enzymes. In Denmark and France, approval is needed prior to use. The information contained in the application dossier necessary for approval should follow the guidelines laid down by the SCF in 1992 or the EU regulation 1332/2008. France has some additional national requirements specified in Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires as amended. In the other EU countries, enzyme preparation should be proved to be safe for use in food before being sold in EU according to the General EU Food Law.

2.2.2 Supporting approvals

T. reesei, including genetically modified strains, has been approved for the production of amylase enzymes, cellulase, glucoamylase, xylanase in the food industry in Denmark and in France. In France, it is also approved for the production of Bêta glucanase and Lysophospholipase (Arrêté du 19 Octobre 2006 as amended).

2.3 Other countries

2.3.1 The enzyme

Aspergilopepsina I (Proteasa ácida fúngica AFP) is positive listed for use as processing aid for food use in general in Mexico. (List: "ACUERDO por el que se determinan los aditivos y coadyuvantes en alimentos, bebidas y suplementos alimenticios, su uso y disposiciones sanitarias."; Link: http://www.cofepris.gob.mx/MJ/Documents/AcuerdosSecretario/acaditivo160712.pdf)

2.3.2 Supporting approvals

T. reseei, including genetically modified strains, has been approved for the production of amiloglucosidase, beta-glucanase, beta-glucosidase, celulase, esterase, hemicelulase and maltase enzymes in the food industry in Brazil (Public Inquiry no. 194 of October 08, 2014, Brazilian Official Gazette of October 08, 2014.).

Strains of *T. reesei* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations" (https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html), as an authorized source for cellulase, glucanase, pentosanase and xylanase in several food applications.

Appendix D1 : Dr Pariza GRAS letter (Mar, 2015)

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7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

March 29, 2015

DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

Dear

I have reviewed the information that you provided on Danisco's *Trichoderma reesei* acid fungal protease (AFP) enzyme preparation, which is produced utilizing an improved strain of *T. reesei*, designated *T. reesei* LOH4 NSP24 #688.1 (GICC03419). The intended uses of the enzyme are for grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of non-citrus juice (i.e., apple juice), the degumming of membranes during orange juice manufacture, and potentially other similar processes, and in fuel ethanol manufacture with resulting distillers grains subsequently used in animal feed.

In evaluating Danisco's AFP enzyme preparation, I considered the biology of *Trichoderma reesei*, relevant information available in the peer-reviewed scientific literature, information that you provided regarding the cloning methodology that was utilized, the safe lineage of the production organism, and results of the safety evaluation studies that Danisco performed on the identical native *T. reesei* AFP enzyme produced by *T. reesei* Morph_NSP24 #22-1 (GICC03243), which was derived from the same intermediate parental strain as *T. reesei* LOH4 NSP24 #688.1 (GICC03419).

By way of background, *T. reesei* is a non-pathogenic, non-toxigenic fungus that is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. Danisco's new AFP production organism, *T. reesei* LOH4 NSP24 #688.1 (GICC03419), was derived by a series of modifications from *T. reesei* QM6a, the original wild-type parental strain used by Danisco to produce a safe strain lineage. Published literature, government laws and regulations, for example

FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and Danisco's unpublished safety studies, all support the conclusion that the lineage to which *T. reesei* LOH4 NSP24 #688.1 (GICC03419) belongs is safe and suitable for use in the manufacture of food-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, xylanase, cellulase, glucoamylase, β-glucosidase/cellulase, alpha-amylase, acid fungal protease, transglucosidase, lipase, and phytase. The enzyme products from 13 production strains within this safe lineage, and in two cases the production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (Regulatory Toxicol. Pharmacol. 33: 173-186, 2001). Some of these enzymes are also the subject of GRAS notification documents that are listed on the FDA GRAS Notice Inventory, for example GRN 00230, 00315, 00333 and 00372, all of which carry the decision statement, "FDA has no questions."

GRN 000333 is of particular relevance to this opinion on the GRAS status of AFP produced by *T. reesei* LOH4 NSP24 #688.1 (GICC03419). The subject AFP enzyme of GRN 000333 was the same native *T. reesei* AFP enzyme preparation, but produced by a different strain (designated *T. reesei* Morph_NSP24 #22-1 (GICC03243), within this safe lineage. Both *T. reesei* Morph_NSP24 #22-1 (GICC03243) and *T. reesei* LOH4 NSP24 #688.1 (GICC03419) were derived from *T. reesei* M1-1.1 (pyr4+), an intermediate strain in the safe strain lineage that was developed from the original wild-type parental strain, *T. reesei* QM6a. In both cases similar genetic procedures were used to generate over-expression of the AFP gene that is native to *T. reesei*. The safety of the AFP enzyme preparation produced by *T. reesei* Morph_NSP24 #22-1(GICC03243) was evaluated with a battery of standard toxicological tests that included acute (14-day) and sub-chronic (91-day) oral toxicity tests in rats, a bacterial reverse mutation assay (Ames test), and two chromosomal aberration tests using human lymphocytes. The AFP test article was not toxic in the acute toxicity study, and no treatment-related effects were observed in the subchronic study at the highest dose tested of 35.8 mg TOS/kg bw/day. The AFP test article did not induce chromosomal aberrations in human lymphocytes, and was not mutagenic in the Ames test.

The AFP enzyme preparation produced by *T. reesei* Morph_NSP24 #22-1(GICC03243) was also assessed for allergenic potential using methods recommended by Codex, which involve searching the primary enzyme structure for homology with known food allergens. Significant homology (greater than 35% identity within multiple 80 amino acid windows) was found only with the respiratory allergen aspergillopepsin (*Asp f 10*) from *Aspergillus fumigatus*. However, *Asp f 10* is not a recognized food allergen, environmental allergens like *Asp f 10* are in general not known to be active via oral exposure, and in any case the actual carry-over of the AFP enzyme preparation into grain and other food/beverage products will be negligible and insufficient to evoke an allergic reaction even if the AFP protein were a food allergen. Based on this information, FDA stated that it had "no questions" with regard to GRAS designation for AFP produced by *T. reesei* Morph_NSP24 #22-1(GICC03243).

Danisco has developed manufacturing conditions and specifications for enzyme manufacture, that are appropriate and suitable for a food-grade ingredient, including the AFP enzyme preparation. Human exposure estimates from the intended uses of the Danisco AFP enzyme preparation in grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of noncitrus juice

(i.e., apple juice), the degumming of membranes during orange juice manufacture, and potentially other similar processes, are well within the generally-accepted (100-fold below NOAEL) safety standard, based on the NOAEL established for AFP produced by *T. reesei* Morph_NSP#22-1 (GICC03243).

The safety of the AFP enzyme preparation produced by *T. reesei* LOH4 NSP24 #688.1 (GICC03419) was formally evaluated using the Pariza-Johnson decision tree. The conclusion of this analysis was that the test article (enzyme preparation) was accepted.

Based on the foregoing, I concur with the evaluation made by Danisco that the *Trichoderma reesei* LOH4 NSP24 #688.1 (GICC03419) production strain is safe to use for the manufacture of food grade acid fungal protease. I further conclude that the acid fungal protease enzyme preparation manufactured using this production strain by the process you described is GRAS (Generally Recognized As Safe) for use in grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of non-citrus juice (i.e., apple juice), the degumming of membranes during orange juice manufacture, and potentially other similar processes, and in fuel ethanol manufacture with resulting distillers grains subsequently used in animal feed.

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

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

Sincerely,

Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

Appendix D2 : **GRAS letter (Sep, 2015)**

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(608) 271-5169
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Michael W. Pariza, Member

September 22, 2015

DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS Opinion on the Intended Uses of DuPont/Danisco's *Trichoderma reesei* acid fungal protease (AFP) enzyme preparation

Dear

I have reviewed the information that you provided on DuPont/Danisco's *Trichoderma reesei* acid fungal protease (AFP) enzyme preparation, which is produced utilizing an improved strain of *T. reesei*, designated *T. reesei* LOH4 NSP24 #688.1 (GICC03419). The intended uses of the enzyme are for grain processing (corn steeping), alcoholic beverage manufacture, non-citrus juice (i.e., apple juice) manufacture, degumming membranes during orange juice manufacture and other similar processes, and fuel ethanol manufacture from grains and cane/molasses with resulting co-products (distillers' grains, corn gluten feed/meal, and vinasse) destined for use in animal feed.

In evaluating Danisco's AFP enzyme preparation, I considered the biology of *Trichoderma reesei*, relevant information available in the peer-reviewed scientific literature, information that you provided regarding the cloning methodology that was utilized, the safe lineage of the production organism, and results of the safety evaluation studies that Danisco performed on the identical native *T. reesei* AFP enzyme produced by *T. reesei* Morph_NSP24 #22-1 (GICC03243), which was derived from the same intermediate parental strain as *T. reesei* LOH4 NSP24 #688.1 (GICC03419).

By way of background, *T. reesei* is a non-pathogenic, non-toxigenic fungus that is used widely by

enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. Danisco's new AFP production organism, *T. reesei* LOH4 NSP24 #688.1 (GICC03419), was derived by a series of modifications from *T. reesei* QM6a, the original wild-type parental strain used by Danisco to produce a safe strain lineage. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and Danisco's unpublished safety studies, all support the conclusion that the lineage to which *T. reesei* LOH4 NSP24 #688.1 (GICC03419) belongs is safe and suitable for use in the manufacture of food-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, xylanase, cellulase, glucoamylase, β-glucosidase/cellulase, alpha-amylase, acid fungal protease, transglucosidase, lipase, and phytase. The enzyme products from 13 production strains within this safe lineage, and in two cases the production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (Regulatory Toxicol. Pharmacol. 33: 173-186, 2001). Some of these enzymes are also the subject of GRAS notification documents that are listed on the FDA GRAS Notice Inventory, for example GRN 00230, 00315, 00333 and 00372, all of which carry the decision statement, "FDA has no questions."

GRN 000333 is of particular relevance to this opinion on the GRAS status of AFP produced by *T. reesei* LOH4 NSP24 #688.1 (GICC03419). The subject AFP enzyme of GRN 000333 was the same native *T. reesei* AFP enzyme preparation, but produced by a different strain (designated *T. reesei* Morph_NSP24 #22-1 (GICC03243), within this safe lineage. Both *T. reesei* Morph_NSP24 #22-1 (GICC03243) and *T. reesei* LOH4 NSP24 #688.1 (GICC03419) were derived from *T. reesei* M1-1.1 (pyr4+), an intermediate strain in the safe strain lineage that was developed from the original wild-type parental strain, *T. reesei* QM6a. In both cases similar genetic procedures were used to generate over-expression of the AFP gene that is native to *T. reesei.* The safety of the AFP enzyme preparation produced by *T. reesei* Morph_NSP24 #22-1(GICC03243) was evaluated with a battery of standard toxicological tests that included acute (14-day) and sub-chronic (91-day) oral toxicity tests in rats, a bacterial reverse mutation assay (Ames test), and two chromosomal aberration tests using human lymphocytes. The AFP test article was not toxic in the acute toxicity study, and no treatment-related effects were observed in the subchronic study at the highest dose tested of 35.8 mg TOS/kg bw/day. The AFP test article did not induce chromosomal aberrations in human lymphocytes, and was not mutagenic in the Ames test.

The AFP enzyme preparation produced by *T. reesei* Morph_NSP24 #22-1(GICC03243) was also assessed for allergenic potential using methods recommended by Codex, which involve searching the primary enzyme structure for homology with known food allergens. Significant homology (greater than 35% identity within multiple 80 amino acid windows) was found only with the respiratory allergen aspergillopepsin (*Asp f 10*) from *Aspergillus fumigatus*. However, *Asp f 10* is not a recognized food allergen, environmental allergens like *Asp f 10* are in general not known to be active via oral exposure, and in any case the actual carry-over of the AFP enzyme preparation into grain and other food/beverage products will be negligible and insufficient to evoke an allergic reaction even if the AFP protein were a food allergen. Based on

this information, FDA stated that it had "no questions" with regard to GRAS designation for AFP produced by *T. reesei* Morph_NSP24 #22-1(GICC03243).

Danisco has developed manufacturing conditions and specifications for enzyme manufacture, that are appropriate and suitable for a food-grade ingredient, including the AFP enzyme preparation. Human exposure estimates from the intended uses of the Danisco AFP enzyme preparation in grain processing (corn steeping), alcoholic beverage manufacture, the manufacture of noncitrus juice (i.e., apple juice), the degumming of membranes during orange juice manufacture, and potentially other similar processes, are well within the generally-accepted (100-fold below NOAEL) safety standard, based on the NOAEL established for AFP produced by *T. reesei* Morph_NSP#22-1 (GICC03243).

The safety of the AFP enzyme preparation produced by *T. reesei* LOH4 NSP24 #688.1 (GICC03419) was formally evaluated using the Pariza-Johnson decision tree. The conclusion of this analysis was that the test article (enzyme preparation) was accepted.

Based on the foregoing, I concur with the evaluation made by Danisco that the *Trichoderma reesei* LOH4 NSP24 #688.1 (GICC03419) production strain is safe to use for the manufacture of food grade acid fungal protease. I further conclude that the acid fungal protease enzyme preparation manufactured using this production strain by the process you described is GRAS (Generally Recognized As Safe) for use in grain processing (corn steeping), alcoholic beverage manufacture, non-citrus juice (i.e., apple juice) manufacture, degumming membranes during orange juice manufacture and other similar processes, and fuel ethanol manufacture from grains and cane/molasses with resulting co-products (distillers' grains, corn gluten feed/meal, and vinasse) destined for use in animal feed.

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

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

Sincerely,

Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

Appendix D3 : GRAS Notice 000333
https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm228952.htm

Agency Response Letter GRAS Notice No. GRN 000333

Return to inventory listing: GRAS Notice Inventory

See also Generally Recognized as Safe (GRAS) and about the GRAS Notice Inventory

CFSAN/Office of Food Additive Safety

September 24, 2010

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

Re: GRAS Notice No. GRN 000333

Dear

The Food and Drug Administration (FDA) is responding to the notice, dated March 29, 2010, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on March 30, 2010, filed it on March 31, 2010, and designated it as GRAS Notice No. GRN 000333.

The subject of the notice is acid fungal protease (AFP) enzyme preparation from *Trichoderma reesei* expressing the gene encoding acid fungal protease from *T. reesei* (AFP enzyme preparation). The notice informs FDA of the view of Genencor, a Danisco Division (Genencor) that AFP enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in grain processing (corn steeping), alcoholic beverage (potable alcohol) manufacture, and degumming of membranes during orange juice manufacturing.

As part of its notice, Genencor includes the report of a panel of individuals (Genencor's GRAS panel) who evaluated the data and information that are the bases for Genencor's GRAS determination. Genencor considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Genencor's GRAS panel evaluated: 1) development of the production organism and safety of the host strain; 2) method of manufacture, product composition, and specifications; 3) intended uses and estimates of dietary exposure; and 4) toxicological studies. The panel stated that there are no reports of disease, toxicity, or allergenicity associated with *T. reesei*. Based on its review, Genencor's GRAS panel concluded that the AFP enzyme preparation is GRAS under the intended conditions of use.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives, or diluents. Enzyme preparations may also contain constituents derived from the production organism and manufacturing process. In their notice, Genencor provides information about each of these components of the AFP enzyme preparation.

Genencor provides specific information about the identity and activity of AFP. According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology (IUBMB), AFP is identified by the Enzyme Commission number 3.4.23.18. Its accepted name is aspergillopepsin I. The Chemical Abstract Service Registry number for AFP is 9025-49-4. AFP is a protease with broad specificity, which primarily catalyzes the hydrolysis of proteins at hydrophobic residues.

Genencor discusses the safety of the production organism, *T. reesei*, which is also known in the literature as *Hypocrea jecorina*. Genencor states that *T. reesei* is nonpathogenic and nontoxigenic based on published literature and its history of use in enzyme production. *T. reesei* has been classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection. BSL1 microorganisms are not known to cause disease in healthy humans.

Genencor describes the development of the *T. reesei* production strain designated NSP24 #22-1. Genencor states that strain NSP24 #22-1 was derived from the host strain RL-P37 by recombinant DNA methods. The RL-P37 strain was developed from the well-known wild-type strain QM6a using classical mutagenesis. Genencor reports that the strain has been determined to be nonpathogenic and nontoxicogenic through intraperitoneal dosing of rats. The *T. reesei* strain RL-P37 has been used by Genencor for over fifteen years for the production of cellulases. Genencor also notes that FDA issued a 'no questions' letter in response to GRN 000230, the subject of which is bovine chymosin obtained from a *T. reesei* production strain that was derived from strain RL-P37.

Genencor states that the host strain RL-P37 was transformed with an expression vector containing the DNA encoding the *T. reesei* mature secreted AFP protein fused to the DNA encoding the *T. reesei* CBH1 signal peptide. Genencor states that the open reading frame is flanked by the promoter and terminator sequences of the *T. reesei cbh1* gene. The expression vector also contains the selectable marker gene *amdS* from *Aspergillus nidulans*. Genencor states that the incorporation of the desired genetic information was evaluated at every step of the construction. The final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *T. reesei* production strain had been made. Genencor states that the production strain is genetically stable, and that no antibiotic resistance genes were used in the development of the production strain.

Genencor states that AFP is produced during cultivation of the production strain using standard fermentation procedures. The fermentation is conducted under controlled conditions and is periodically monitored for microbial contamination. AFP is secreted to the fermentation broth and is subsequently recovered via several purification and concentration steps. The resulting liquid concentrate (AFP concentrate) is stabilized with food-grade glycerol, sodium sulfate, and sodium benzoate, and is tested to demonstrate it meets product specifications. The final enzyme preparation contains 2.8-3% AFP and 5.1-5.5% total organic solids (TOS). Genencor states that the manufacturing process is performed in accordance with current Good Manufacturing Practice (cGMP) as set forth in 21 CFR Part 110. Genencor states that the AFP enzyme preparation conforms to the specifications for enzyme preparations described in the Food Chemicals Codex (6th edition) and to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (2006).

Genencor states that the AFP enzyme preparation will be used in grain processing at 9 micrograms (µg) per gram (g), in alcoholic beverage manufacturing at 160 ug per milliliter, and in degumming equipment membranes in orange juice manufacturing. To estimate human exposure to the AFP enzyme preparation, Genencor assumes that AFP enzyme preparation would be used at the highest intended use levels for grain processing and alcoholic beverage manufacturing and that 100% of the enzyme would be carried over to the final food product. In actuality the carryover of AFP enzyme preparation will be negligible. Genencor states that AFP used in degumming filter membranes during orange juice processing is not expected to be carried over to the juice because the membranes are extensively washed after degumming prior to re-use in the juice production. Given these assumptions, the cumulative exposure to AFP enzyme preparation would be 147 µg of TOS per kilogram bodyweight per day (µg TOS/kg bw/d)

Genencor summarizes toxicological studies conducted with the AFP concentrate containing 14.3 percent TOS. The studies include acute (14-day) and sub-chronic (91-day) oral toxicity tests in rats, a bacterial reverse mutation assay (Ames test), and two chromosomal aberration tests using human lymphocytes. Genencor concludes that the AFP concentrate was not toxic in the acute toxicity study and that no treatment-related effects were observed in the subchronic study at the highest dose tested of 35.8 mg TOS/kg bw/d. Genencor also states that the AFP concentrate did not induce chromosomal aberrations in human lymphocytes and has not shown mutagenic activity in the Ames test.

Genencor provides information regarding the potential allergenicity of AFP and of enzymes used in food processing in general. Consistent with the recommendations of the Codex Alimentarius Commission (2003), Genencor performed an amino acid sequence homology search for AFP against known allergens listed in the publicly available Structural Database of Allergenic Proteins (SDAP). Genencor identified a significant homology (> 35% identity within multiple 80 amino acid windows) with the respiratory allergen aspergillopepsin (Asp f 10) from *Aspergillus fumigatus*. The notifier subsequently analyzed the hydrophilicity of AFP and concluded that none of the matching sequences is hydrophilic and therefore would not be exposed on the surface of a folded protein where it could act as an immunogenic epitope. Genencor also notes that Asp f 10 is not a recognized food allergen and that environmental allergens are generally not known to be active via oral exposure. Further, Genencor states that the actual carry-over of AFP into products from grain processing would be negligible and insufficient to invoke an allergic reaction even if the AFP protein were a food allergen.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FFDCA to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Genencor's notice that the AFP enzyme preparation is GRAS for the intended use, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing AFP enzyme preparation. Accordingly, this response should not be construed to be a statement that foods that contain the AFP enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Genencor, as well as other information available to FDA, the agency has no questions at this time regarding Genencor's conclusion that the AFP enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of AFP enzyme preparation. As always, it is the continuing responsibility of Genencor to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000333, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying via the FDA home page at http://www.fda.gov. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Sincerely,

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Appendix D4 : France Authorization of Aspergillopepsin I from *T.reesei* (Confidential Commercial Information)

Please refer to Appendix D Confidential Attachment

Appendix D5 : Denmark Authorization of Aspergillopepsin I from *T.reesei* (Confidential Commercial Information)

Please refer to Appendix D Confidential Attachment

Appendix D6 : EU Legislation on Enzymes

Amfep/09/01 — Association of Manufacturers and Formulators of Enzyme Products — 18 January 2009

Amfep Statement on the EC Regulation 1332/2008 on Food Enzymes

On 16 December 2008 the European Parliament and the Council adopted Regulation 1332/2008 EC on food enzymes (OJ EU L 354/7, 31.12.2008).

The Association of Manufacturers and Formulators of Enzyme Products (Amfep) welcomes this EU legislation which is the first attempt ever to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU. The Regulation is set to improve the functioning of the internal market by removing disparities among member states and bringing more legal certainty to the market.

Only authorised food enzymes will be allowed to be commercialised and/or used in foods sold in the EU – irrespective whether they are used as processing aids or ingredients. This also applies to imported foods. The European Food Safety Authority (EFSA) will play a pivotal role in the authorisation process of food enzymes. On the basis of EFSA's scientific advice, the EU Commission will grant authorisations after consulting member states and the EU Parliament.

The publication of the Regulation in the Official Journal of the European Union only marks the beginning of an extensive implementation process. In fact, several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable.

The European Commission, supported by EFSA, has until 2011 to specify what information is required to be submitted for a risk assessment of food enzymes. After that, the industry will have another two years (until 2013) to submit dossiers for evaluation and authorization of food enzymes presently used in food on the EU market. Only after the EU Commission and EFSA have completed the evaluation of all these dossiers will the first EU (positive) list of approved food enzymes be established. The Regulation 1332/2008 EC does not set a deadline by which this evaluation is to be completed.

Apart from the authorization requirements, Regulation 1332/2008 also lays down specific provisions on labelling of food enzymes, food enzyme preparations and food prepared with enzymes. The provisions on labelling of food enzymes and food enzyme preparations will enter into force on 20 January 2010, whereas the provisions on labelling of food prepared with enzymes enter into force on 20 January 2009. The latter do not increase the scope of the previous food labelling provisions, although some changes are introduced to the way the small number of food enzymes that are not used as processing aids are declared.

Amfep is working closely with relevant European Stakeholders to ensure a seamless implementation of the new EU legislation for the benefit of food enzyme manufacturers, their clients, and consumers.

NB: The proposal for a regulation on food enzymes is a part of a so-called Food Improvement Agents package (FIAP). While harmonising EU legislation for food enzymes, FIAP is also aiming at upgrading existing EU legislation on food additives (EC Regulation 1333/2008) and food flavourings (EC Regulation 1334/2008) and establishing a transversal authorisation procedure (EC Regulation 1331/2008) The EU Regulation on food additives will include a positive list of additives and carriers that will be allowed in food enzymes and food enzyme preparations. This list will come into force on 1 January 2011.

For more information contact: Youri Skaskevitch
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