A Xylanase Enzyme
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 Biosciences (IB) is seeking approval for a "Xylanase (EC 3.2.1.8)" enzyme for use as processing aid in bakery application. The enzyme is designated as "Xylanase" throughout the dossier.

The enzyme Xylanase is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress the xylanase gene from *Aspergillus niger (var. tubingensis).*

The enzyme is intended for use in baking for the production of bread, buns, cakes, sweet goods and tortillas and other bakery products. In baking, Xylanase performs its technological function during the dough or batter handling to improve the dough stability and dough handling properties.

In all of these applications, Xylanase 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 Xylanase for use in these applications, Dupont IB vigorously applied the criteria identified in the guidelines as laid down by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilizing enzyme toxicology/safety data, the safe history of use of enzyme preparations from *T. reesei* and of other xylanase enzymes in food, the history of safe use of the *T. reesei* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

In addition, different endpoints of toxicity were investigated, and the results are evaluated and assessed in this document. In genotoxicity studies, Xylanase is not mutagenic, clastogenic or aneugenic. Daily oral administration of Xylanase up to and including a dose level of 1000 mg total protein/kg bw/day or 1214.4 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming Xylanase from baking application, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.488 mg TOS/kg body weight/day. This still offers a 2489 fold margin of safety.

Based on the results of safety studies and other evidence, Xylanase has been demonstrated as safe for its intended applications and at the proposed usage levels. Approval of this application would provide manufacturers and/or consumers with benefits of facilitating the baking process, lowering the manufacturing cost, and improving quality of final foods.

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

(c) Address:

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

(e) Email Address:

See above

(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 to Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application. The intended use of the processing aid is baking.

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.

Xylanase, subject of this application, is intended for use in baking, for the production of bread, buns, cakes, sweet goods and tortillas and other bakery products.

Currently no Xylanase from *A. niger (var. tubingensis)* expressed in *T. reesei* is permitted as a Processing Aid, however Xylanase from *T. reesei*, and other enzymes including Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, 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

Xylanase is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the Xylanase gene from *A. niger* var. *tubingensis.* The enzyme is characterized as an Endo-1,4-beta-xylanase (EC 3.2.1.8). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

The enzyme is intended for use in the baking for the production of bread, buns, cakes, sweet goods and tortillas and other bakery products. In baking, Xylanase performs its technological function during the dough or batter handling to improve the dough stability and dough handling properties.

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

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no xylanase from *A. niger (var. tubingensis)* expressed in *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 Endo-1,4-beta-xylanase from *A. niger (var. tubingensis)* expressed in *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 Xylanase derived from *T. reesei* containing the gene for Xylanase from *A. niger (var. tubingensis)* in the Australia/New Zealand market. 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 to 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 (CCI)

Certain (identified) technical and manufacturing information included in Appendices B1, B3, -B6, Appendices D1, D3, Appendices E1-E5 and other information including amino acid sequences labelled with Confidential Commercial information 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 Commercial Capturable 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

Xylanase from *A. niger (var. tubingensis)* 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

Xylanase derived from *T. reesei* carrying the gene encoding the xylanase gene from *A. niger (var. tubingensis)* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in production of bakery products by a panel of scientific experts in the USA.

1.9. Statutory declaration

of ,

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: _____

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

Nutritive Substances (3.3.3)

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

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Xylanase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the xylanase gene from *A. niger* var. *tubingensis*.

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

2.2.1 Chemical/Common Name:

The systematic name of the principle enzyme activity is 4-β-D-xylan xylanohydrolase. Other names used are endo-1,4-β-xylanase, endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase.

- EC number: 3.2.1.8
- CAS number: 9025-57-4

Biological source: The Xylanase enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei*, carrying the xylanase gene from *Aspergillus niger (var. tubingensis)*.

2.2.2 Marketing Name of the Processing Aid:

The marketing name of this enzyme preparation will depend on the application. An example marketing name of Xylanase is POWERbake® 8200.

2.2.3 Molecular and Structural Formula:

Xylanase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

The function of Xylanase is to catalyse the hydrolysis of the (1-4)-beta-D-xylosidic linkages that are present in the centre (endo) of xylans, including arabinoxylan.

When added to bread dough under controlled conditions, Xylanase will partially degrade the hemicellulose network of the dough. Hemicellulose, including arabinoxylans, provides functional properties during bread making due to its ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of the hemicellulose network and water-unextractable (WU) arabinoxylans hampers the optimal use of the natural constituents of the dough in the baking process. The use of a xylanase results in partially solubilised hemicellulose and arabinoxylans with lower molecular weights, which increases the water binding capacity of the dough and improves the functional baking properties of these polysaccharides, resulting in:

- better handling of the dough (improved extensibility and stability, reduced stickiness leading to reduced losses of dough)

- improvement in dough's structure and behaviour during the baking step
- more uniform and slightly increased volume and an improved crumb structure of the bakery product, which might otherwise be impaired by processing of the dough
- reduced batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

Substrate specificity:

The function of Xylanase is to catalyse the hydrolysis of the (1-4)-beta-D-xylosidic linkages that are present in the centre (endo) of xylans, including arabinoxylan. The substrates for Xylanase are xylans, including arabinoxylans.

Activity:

The activity of the Xylanase is defined in GPU. The substrate employed in the assay is azurine-crosslinked wheat arabino-xylan. This substrate hydrates in water but is water insoluble. Hydrolysis of the substrate by an endo-xylanase produces water-soluble fragments, and the rate of these fragments can be related directly to the enzyme activity by spectroscopy.

Temperature optimum:

Approximately 50°C, with relatively high activity from 45 to 55°C.

Thermal stability:

The enzyme activity dropped to below 10% after 10 minutes of incubation at 65°C.

pH optimum:

Approximately pH 4.0, with high relative activity at pH interval 3.5-4.5.

pH stability:

Optimal stability is seen at the pH interval 3.5-4.5 and the enzyme activity is observed in the pH range 3.5-5.5.

Interaction of the enzyme with different foods:

The Xylanase 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:

Xylanase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Xylanase are very low, and as with other enzymes that are currently approved and used as Processing Aids, use of this preparation 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 Xylanase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation 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 Xylanase to ensure that the finished preparation 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 10,000 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	Negative by test
Physical properties:	
Appearance	Off white powder

Standard for identity:

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

2.6. Allergenicity of the enzyme:

Bioinformatic analyses based on sequence homology determined that the *Aspergillus niger* (var. *tubingensis*) xylanase is unlikely to pose a risk of food allergenicity. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

An allergen statement is given in Appendix A9. Wheat may be present in the final preparation. As this enzyme is used for bakery purpose, the presence of wheat in the enzyme preparation is not expected to introduce any additional food allergen risk to consumers.

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 Xylanase shows a clear conserved Glyco_hydro_11 superfamily sequence domain, characteristic for endo-1,4-beta-xylanase activities.

Xylanase enzyme, the subject of this dossier, is one of the approved endo-1,4-xylanase enzymes on Schedule 18 of the ANZ Food Standards Code, i.e. the *Aspergillus niger* one. In our case the enzyme protein is expressed from *Trichoderma reesei*. The identity between the FSANZ approved endo-1,4-xylanase (*A. niger, A.oryzae, A. aculeatus, T. lanuginosus, B. amyloliquefaciens, B. subtilis, B. licheniformis, H. insolens, T. reesei*) ranges from 16.9 - 75.5%. It is good to realize that the endo-1,4-xylanase sequences within one species can show strain dependent amino acid sequence variability. Also, several microorganism species contain more

than one endo-1,4-xylanase encoding genes with different sequences (e.g. *H. insolens* XynA and XynB).

Xylanase enzyme derived from *T. reesei*, carrying the xylanase gene from *A. niger (var. tubingensis)* has been determined to be GRAS in the United States, and been used for bread and other bakery applications in multiple countries like Norway, Peru since 2016. There have not been any adverse events reported since Xylanase 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

Toxin homology study

A BLAST search for homology of the xylanase sequence against the complete Uniprot database (http://www.uniprot.org/), was performed, with a threshold E-value of 0.1. The majority of matches were endo-1,4-beta xylanases, with none of the top 1000 database matches being annotated as either toxin or venom.

In addition, a specific BLAST search for homology of the mature xylanase sequence was performed against the Uniprot animal toxin database. This yielded no matches.

Therefore, the xylanase sequence does not share homology with a known toxin or venom sequence.

Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly 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.

Although *T. reesei* is scientifically determined by DuPont IB as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on *T. reesei* LOVXLNA #568.4 are thus one of the pillars supporting the DuPont IB *T. reesei* Safe Strain Lineage. The position of the food enzyme in the DuPont IB *Trichoderma reesei* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

To assess the safety of Xylanase, different endpoints of toxicity were investigated and are evaluated and assessed in this document:

- 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 NOAEL (no observed adverse effect level) is established at the highest dose tested, 1000 mg total protein/kg bw/day, equivalent to 1214.4 mg total organic solid (TOS)/kg bw/day in male and female rats.

A summary of the results of the studies can be found in Appendix B.

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 strain LOVXLNA #568.4 is a strain of *T. reesei* which has been genetically modified by DuPont IB to overexpress a xylanase gene from *A. niger* var. *tubingensis*.

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), 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 preparations 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). The xylanase gene was placed under the expression signals of the endogenous *T. reesei cbh1* gene, and 2-7 copies of the expression cassette were integrated into the recipient genome, using the endogenous *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker.

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

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.

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. A review of the literature search uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals.

Strain QM6a and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme preparations 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 preparations by Food Standards Australia New Zealand, and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan. Further details are discussed in Appendix B.

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, and has demonstrated 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 as confirmed by genome sequencing. Refer also section 3.6.

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

The production organism of the Xylanase preparation, the subject of this submission, is *T. reesei* strain LOVXLNA #568.4. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance xylanase 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 *A. niger* var. *tubingensis*. Xylanase expression cassette was integrated into the host genome. Full details of the genetic modifications are provided in Appendix E2 (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by genome sequencing. Broth samples were taken prior and after prolonged fermentation mimicking commercial fermentation conditions. Samples were then used for genomic DNA extraction and next generation sequencing. A complex integration site for xylanase expression site was determined, and no change was observed between samples prior and after fermentation. The results demonstrate that the insertion cassette has been stably maintained through generations during the fermentation process.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E1-E3. 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), Xylanase will be used in:

- 7. Bread and Bakery Products

4.2. Levels of residues in food

The proposed application rate of Xylanase in its intended application is listed below.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	0.15 – 55	55

DuPont IB expects the Xylanase to be inactivated or removed during the subsequent production and refining processes for all applications.

In baking, Xylanase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. The Xylanase is denatured by heat during the baking or steaming step.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables one 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.

Based on the raw materials used in the various food processes, the recommended use levels of the enzyme Xylanase, for the calculation of the TMDI, the maximum use levels are chosen. The TMDI is calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day. Consequently, the TMDI will be: 0.488 mg TOS/kg body weight/day. The NOAEL has been determined for Xylanase to be at 1000 mg total protein/kg bw/day (equivalent to 1214.4 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 2489 fold margin of safety. It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value. Please refer to Appendix C for details.

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 enzyme would be used as a processing aid in about:

- 20% of the tonnage of bread and bakery products sold in Australia and New Zealand

4.4. Levels of residues in food in other countries

Applications and levels of use of the Xylanase preparation in other countries is the same as presented in section 4.2.

5. References

Bissett J (1984). A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. nov. Canadian Journal of Botany, 62(5), 924-931

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology, 39(2), 214-228

Brückner H, Graf H (1983). Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma ressei* Simmons Part A.Experientia, 39(5), 528-530

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

Hansen, S.C. (1996). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet. Toxicol., 4, 427-432.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Khuls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, Petrini O, Gams W, Börner T, Kubicek CP (1996). Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*, Proc. Natl. Acad. Sci. USA 93, 7755-7760

Mandels M and Reese ET (1957). Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. J Bacteriol. 73, 269-278

Meyer W, Morawetz R, Borner T, Kubicek CP (1992). The use of DNA-fingerprint analysis in the classification of some species of the Trichoderma aggregate. Curr. Genet. 21, 27-30

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

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006). Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharmacol 45, 144-158

Pariza,MW, Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing – Update for a New Century. Regul Toxicol Pharmacol, 33(2), 173-86,

Solfrizzo M, Altomare C, Visconti A, Bottalico A, Perrone G (1994). Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. Natural toxins, 2(6), 360