A Pullulanase Enzyme Preparation from a recombinant strain of *Bacillus licheniformis*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

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

May 17, 2018

CONTENTS:

1. General information 2
1.1 Applicant details 2
1.2 Purpose of the application 3
1.3 Justification for the application 3
1.4 Support for the application 4
1.5 Assessment procedure 4
1.6 Confidential Commercial Information 4
1.7 Exclusive capturable commercial benefit (ECCB) 4
1.8 International and other National Standards 4
1.9 Statutory declaration 6
1.10 Checklist 7
2. Technical information 9
2.1. Type of processing aid 9
2.2. Identity 9
2.3. Chemical and physical properties 9
2.4. Manufacturing process 11
2.5. Specification for identity and purity 11
3. Safety 13
3.1. Use of the enzyme as a food processing aid in other countries 13
3.2. Toxicity of the enzyme 14
3.3. Information on the source micro-organism 15
3.4. Pathogenicity and toxicity of the source micro-organism 15
3.5. Genetic stability of the source organism 15
3.6. Method used in the genetic modification of the source organism 15
4. Dietary exposure 16
4.1. List of food or food groups likely to contain the enzyme or its metabolites 16
4.2. Levels of residues in food 16
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 17
4.4. Levels of residues in food in other countries 17
5. References 18

APPENDIX A: Technical information

APPENDIX B: Safety

APPENDIX C: Dietary exposure

APPENDIX D: International and other National Standards

APPENDIX E: Information on the methods used in the genetic modification of the source organism (Confidential Commercial Information)

EXECUTIVE SUMMARY:

DuPont Industrial Bioscience (IB) is seeking approval for a pullulanase enzyme product for use in converting pullulan and amylopectin in the processing of food raw material which naturally contains the substrate. The enzyme is herein designated as Pullulanase.

The Pullulanase preparation which is the subject of this submission is derived from a pure culture of a non-pathogenic, non-toxigenic, and asporogenic strain of *Bacillus licheniformis,* BMP139. BMP139 is a genetically modified strain that has been constructed to express a pullulanase gene derived from a non-pathogenic and non-toxigenic microorganism *Bacillus deramificans*.

The food enzyme object of the dossier is typically used in brewing and starch processing,

In all of these applications, Pullulanase 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 Pullulanase for use in brewing and starch processing, DuPont IB vigorously applied the criteria identified in the guidelines laid out by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilizing enzyme safety data, the safe history of use of other enzyme preparations from *B. licheniformis* and of other pullulanases 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.

To assess the safety of Pullulanase produced by *B. licheniformis* BMP 139 in foods, DuPont IB investigated different endpoints of toxicity through studies conducted at BioReliance (Maryland) and ClinTrials (Canada). These studies are evaluated and assessed in this document. The toxicology studies included:

- Ames mutagenicity studies
- I*n vitro* Chromosomal Aberration Study
- Sub-chronic 90-day toxicity study in the rat

Based on conservative assumptions and a highly exaggerated value consumption data, the NOAEL still offers a 4837× fold Margin of Safety.

Based on the results of safety studies and other evidence, Pullulanase 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 brewing process and starch processing, potentially lowering the manufacturing cost, and improving quality of final foods.

1. 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:
Level 3, 7 Eden Park Drive, Macquarie Park,
NSW 2113. Locked Bag 2067 North Ryde BC
NSW 1670, Australia

(d) Contact Details:

Axiome Pty Ltd
PO Box 150 Blackheath NSW 2785, Australia

Danisco Singapore Pte Ltd
21 Biopolis Road #06-21
Nucleos, South Tower
Singapore 138567

(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.

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 Australian food manufacturer.

Currently no pullulanase from *Bacillus deramificans* expressed in *Bacillus licheniformis* is permitted as a Processing Aid. However other enzymes including α-amylase, Pullulanase, and Serine Proteinase from *B. licheniformis* are listed in Schedule 18 Section S18-4(5) as permitted enzymes. *B. licheniformis* recombinant strains are also listed in Schedule 18 Section S18-4(5) as production organism for α-Amylase, Chymotrypsin, Endo-1,4-beta-xylanase, β-Galactosidase, Glycerophospholipid cholesterol acyltransferase, and Maltotetraohydrolase.

Pullulanase subject of this application is intended for use in brewing and starch processing.

Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Sections 1.3, 2.3 and Appendix A.

1.3 Justification for the application

The need for the proposed food regulatory measure and the advantages of the proposed change are covered in Section 1.2.

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application

The Pullulanase is an enzyme preparation produced by submerged fermentation of *B. licheniformis* carrying the gene encoding a pullulanase from *B. deramificans.* The enzyme is characterized as pullulanase (EC 3.2.1.41). 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.

The effect of the Pullulanase is the conversion during food processing of pullulan and amylopectin in starch containing raw materials with the release of oligosaccharides and glucose, resulting in improved processing properties of these materials. The main intention of the use of Pullulanase is to facilitate the degradation of starch in brewing and starch processing. However, depending on the specific application, the enzymatic hydrolysis of starch with the help of Pullulanase can result in some benefits on the final foods.

More information on the benefit of this enzyme can be found in Section 2.3 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no pullulanase from *B. deramificans* expressed in *B. licheniformis* 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 Pullulanase 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 the Pullulanase expressed in *B. licheniformis* containing the gene for pullulanase from *B. deramificans* in Australian and New Zealand. 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

Certain (identified) technical and manufacturing information included in Appendices B1, B2, B3, B4, B5 Supplementary 1, Appendix D3, Appendix E and other information including but not limited to 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 capturable commercial benefit (ECCB)

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

1.8 International and other National Standards

Refer to Appendix D for further details and documentation confirming international regulatory status

1.8.1. Codex Standards

Pullulanase from *B. deramificans* expressed in *B. licheniformis* has been reviewed by JECFA; JECFA specification of the Pullulanase is included in Appendix D1.

1.8.2. International Legislation

Pullulanase from *B. deramificans* expressed in *B. licheniformis* has been determined to be GRAS in the United States as a food processing aid in carbohydrate processing, brewing, potable alcohol and fuel ethanol manufacture by a panel of scientific experts in the USA. It is also the subject of GRAS Notice 000072 with a concurrence letter received from FDA, dated

Jun 12, 2001. It is also approved for the production of glucose syrup and potable alcohol in both France and Denmark. Refer Appendix D.

1.9 Statutory declaration

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

Food Additives (3.3.1)

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

Processing Aids (3.3.2)

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

Refer to Appendix A for further details.

2.1. Type of processing aid

The Pullulanase is an enzyme preparation produced by submerged fermentation of *B. licheniformis* carrying the gene encoding a pullulanase enzyme from *B. deramificans.*

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

2.2. Identity

a) Chemical/Common Name:

The systematic name of the principle enzyme activity is pullulan 6-α-glucanohydrolase. Other names used are limit dextrinase (erroneous); amylopectin 6-glucanohydrolase; bacterial debranching enzyme; debranching enzyme; α-dextrin endo-1,6-α-glucosidase; R-enzyme; pullulan α-1,6-glucanohydrolase.

- EC number: 3.2.1.41
- CAS number: 9075-68-7

Biological source: The Pullulanase is an enzyme preparation produced by submerged fermentation of *B. licheniformis* carrying the gene encoding a pullulanase enzyme from *B. deramificans.*

b) Marketing Name of the Processing Aid:

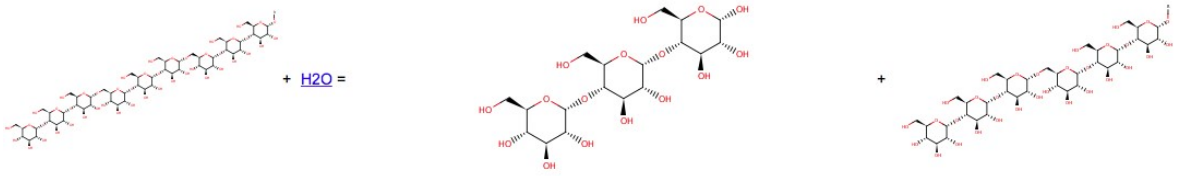
Not all marketing names for the pullulanase preparation subject of this application has been finalised at this stage. However, depending on the application, the marketing name of the processing aid could be Optimax® L-1000.

c) Molecular and Structural Formula:

Pullulanase is a protein. The amino acid sequence is known. Refer to Section 3.3. and Appendix E.

2.3. Chemical and physical properties

Pullulanase (IUBMB EC.3.2.1.41) hydrolyses (1->6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen.



(Glcalpha(1-4)Glcalpha(1-4)Glcalpha(1-6))n + H2O = alpha-D-glucopyranosyl-(1-4)-alpha-D-glucopyranosyl-(1-4)-alpha-D-glucopyranose + (Glcalpha(1-4)Glcalpha(1-4)Glcalpha(1-6))n-1

The main intention of the use of Pullulanase is to facilitate the degradation of starch in brewing and starch processing. However, depending on the specific application, the enzymatic hydrolysis of starch with the help of Pullulanase can result in some benefits on the final foods. In brewing, the main intention of the use of Pullulanase is to facilitate the brewing process by increasing the amount of fermentable sugars. In starch processing, the main intention of the use of Pullulanase is to facilitate the production of glucose and maltose syrups from starch.

In all of these applications, the 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.

Appearance: The commercial enzyme preparation is an amber coloured liquid.

Substrate specificity:

Pullulanase (IUBMB EC.3.2.1.41) hydrolyses (1->6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen. Substrates include pullulan, amylopectin and glycogen.

Activity: The activity of the pullulanase is defined in ASPU: Acid Stable Pullulanase Units. This unit is not defined in exact terms, but relies on a specific assay and an enzyme standard. The assay is based on a soluble Red Pullulan substrate purchased from the company Megazyme (Appendix A). The specific activity of a commercial Pullulanase product is 1200 ASPU/g enzyme protein using this assay.

Temperature optimum: approximately 52-62°C (See also Appendix A, Section 2.3).

Thermal stability: The enzyme exhibits activity from 30°C until 80°C. The activity of the enzyme is rapidly decreasing with temperatures above 60°C. No enzyme activity is left at temperatures above 67°C. (See also Appendix A, Section 2.4.)

pH optimum: approximately pH 4-5. (See also Appendix A, Section 2.5.)

pH stability: Optimal stability is seen at the pH interval 3.0 to 7.5 and the enzyme is relatively stable in the pH range 3.2-6.7. (See also Appendix A, Section 2.5.)

Interaction of the enzyme with different foods:

In all of these applications, the 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

Pullulanase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Pullulanase 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 Pullulanase 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 Pullulanase 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 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, amber

Standard for identity:

Pullulanase 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, pullulanase preparation subject of this application do resemble pullulanase 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 types. 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 shows clear conserved pullulanase sequence domains (including the 'PulA' super domain and 'CMB41_Pullulanase' domain), characteristic for pullulanase (IUBMB 3.2.1.41) enzymes. Our pullulanase sequence shows 64% identity to the pullulanase from *B. acidopullulyticus*, which is one of the approved pullulanase enzymes on Schedule 18 of the FSANZ Code. The identity among the FSANZ approved pullulanases range from 29% (*B. acidopullulyticus* to *K. pneumoniae*) to 94% (*B. amyloliquefaciens* to *B. licheniformis*). Note that even available pullulanase sequences obtained from different strains of one species show variability. For instance, an alignment of just four of the available *B. licheniformis* pullulanase amino acid sequences showed that these were 98-100% identical.

Pullulanase derived from *B. licheniformis* carrying the gene encoding a pullulanase from *B. deramificans* has been determined to be GRAS in the United States. It is also the subject of GRAS Notice 000072 with a concurrence letter received from FDA, dated Jun 12, 2001. Pullulanase has been evaluated by JECFA, Denmark and France. Pullulanase has been used in U.S. since 1999 in saccharification in starch processing, and for similar purpose in Europe, Latin America, China,and other Asian countries afterwards. There have not been any adverse events reported since Pullulanase 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 *B. licheniformis* BMP139 is safe as a production organism as it pertains to the DuPont *B. licheniformis* Safe Strain Lineage (see Appendix B1).

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 Solids (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 *B. licheniformis* is scientifically determined by DuPont IB as a Safe Strain Lineage, the food enzyme subject of the current dossier is supported by toxicological studies on the specific food enzyme subject of this dossier. The toxicological studies on *B. licheniformis* Pullulanase BMP 139 are thus one of the pillars supporting the DuPont IB *B. licheniformis* Safe Strain Lineage. The position of the food enzyme in the DuPont IB *B. licheniformis* Safe Strain Lineage is presented in Appendix B1.

Toxicological testing

To assess the safety of Pullulanase produced by *B. licheniformis* BMP 139 in foods, DuPont IB investigated different endpoints of toxicity through studies conducted at BioReliance (Maryland) and ClinTrials (Canada). The toxicology studies included:

- Ames mutagenicity studies
- I*n vitro* Chromosomal Aberration Study
- Sub-chronic 90-day toxicity study in the rat

These studies are evaluated and assessed below.

The safety of Pullulanase has been assessed in toxicology studies investigating its mutagenic and systemic toxicity potential. In genotoxicity studies, Pullulanase is not mutagenic, clastogenic or aneugenic. Daily oral administration of Pullulanase up to and including a dose

level of 169 mg total protein/kg bw/day or 237 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects. 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 of the Pullulanase preparation, the subject of this submission, is *B. licheniformis* strain BMP139. It is derived by recombinant DNA methods from strain Bra7. The purpose of this genetic modification is to express the gene encoding a pullulanase from *B. deramificans*. Bra7 is a classical industrial strain used for α-amylase production by DuPont IB and its parent companies since 1989.

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

The host species *B. licheniformis* is an accepted source of safe food enzymes in the literature. *B. licheniformis* has a long history of safe use in industrial-scale enzyme production. The long industrial use and wide distribution of *B. licheniformis* in nature has never led to any pathogenic symptoms. Moreover, no case demonstrating invasive properties of the species has been found in the literature. The safety of *B. licheniformis* strains was reviewed (De Boer, A.S., *et al.* 1994.). Pathogenic strains are not described in the Bergey's Manual of Systematic Bacteriology or in the ATCC and other catalogues. The species *B. licheniformis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work".

The non-pathogenicity of the donor organism, *B. deramificans* pullulanase gene was well established and documented in pathogenicity studies of a recombinant *B. licheniformis* strain containing a pullulanase gene. *B. deramificans* is classified as belonging to Risk Group I by the German authorities ("Zentralen Kommision für die Biologische Sicherheit (ZKBS)"), and a GMO production strain consisting of a *B. licheniformis* host and an integrated plasmid carrying the same *B. deramificans* pullulanase gene was also classified as belonging to Risk Group I by the ZKBS (document 6790-01-1002, dated 26/8/93).

3.5. Genetic stability of the source organism

The parental strain of the production strain *B.licheniformis* Bra7 and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont IB and its parental companies, and stable enzyme expression even at large scale fermentation has been observed. Please also refer to Appendix B1 for list of example enzyme preparations produced using Bra7 and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable after more than 60 generations of fermentation for pullulanase production. Refer also section 3.6.

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

The production organism of Pullulanase preparation, the subject of the submission is *B. licheniformis* strain BMP139. It is derived by recombinant DNA methods from strain Bra7. The purpose of this genetic modification is to express pullulanase from *B. defamificans*, and deleted two endogenous proteases. Chromosomal deletions of specific genes were conducted by well-known techniques (Stahl & Ferrari 1984). The only heterologous DNA contained in BMP139 is the pullulanase expression cassette, which is integrated into the chromosome of BMP139 to achieve a high degree of stability.

The donor organism is *B. deramificans*.

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 pullulanase 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 genetic stability analysis 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 (S15-5), Pullulanase will be used in:

- 14.2.1 Beer and related products
- 11.2 Sugars and sugar syrups and 11.4 Tabletop sweeteners

4.2. Levels of residues in food

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

- Brewing
- Starch processing

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

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:

0.049 mg TOS/kg body weight/day.

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:

- 50% of beer sold in Australia and New Zealand
- 20% of sweetener sold in Australia and New Zealand

4.4. Levels of residues in food in other countries

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

5. References

De Boer AS, Priest F, Diderichsen B (1994). On the industrial use of *Bacillus licheniformis*: a review. Appl. Microbiol. Biotechnol 40, 595-598

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

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,

Sietske de Boer A, Priest F and Diderichsen B (1994). On the industrial use of *Bacillus licheniformis*: a review. Appl Microbiol Biotechnol, 40, 595-598

Stahl ML and Ferrari E. (1984). Replacement of Bacillus subtilis Subtilisin Structural Gene with an In Vitro-Derived Deletion Mutation. J Bacteriol, 158 (2), 411-418