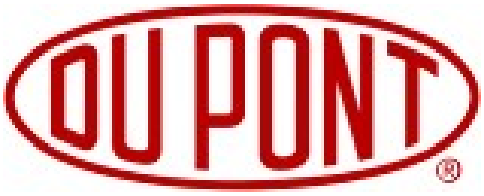

A Lactase Enzyme
from a recombinant strain of *Bacillus subtilis*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DUPONT AUSTRALIA PTY LTD
Submitted by:

Jun 7, 2018

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EXECUTIVE SUMMARY:

DuPont Industrial Biosciences (IB) is seeking approval for a lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) for use as a processing aid in dairy processing and in the production of galacto-oligosaccharide (GOS). The enzyme is designated as "CB108 Lactase" throughout the dossier.

CB108 Lactase enzyme preparation is produced by submerged fermentation of *Bacillus subtilis* carrying the lactase gene from *Bifidobacterium bifidum* encoding the wild-type truncated lactase enzyme.

CB108 Lactase enzyme is intended for use in dairy processing for production of lactose reduced dairy products including but not limited to milk, yogurt, cheese, and the production of galacto-oligosaccharides (GOS). The CB108 Lactase is also considered a β-galactosidase because it is involved in the hydrolysis of lactose to galactose and glucose. In dairy processing, the enzyme will convert lactose into GOS and glucose. CB108 Lactase will be able to generate GOS in situ in raw milk or whey even with low lactose content (roughly 5% lactose content in milk), with as benefit to provide a low lactose/lactose free dairy product with reduced total sugars and caloric content in the final dairy product and enable dairy products to contain GOS prebiotic material. The enzyme will also be used in the production of purified GOS.

In all of these applications, CB108 Lactase 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 technical function in the final food.

To assess the safety of the CB108 Lactase 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 *B. subtilis* and of other lactase enzymes in food, the history of safe use of the *B. subtlis* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

The safety of the food enzyme from *B. subtilis* has been assessed using toxicology studies conducted on earlier strains of the DuPont *B. subtilis* 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:

- Acute oral toxicity test: not acute toxic under the test condition. The oral LD_{50} for in female rats was greater than 5000 mg/kg bw/day
- 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 total protein/kg bw/day, which is the high dose in the study. This NOAEL is equivalent to 1,416.4 mg total organic solids (TOS)/kg body weight/day.

Based on a conservative assumption and a highly exaggerated value consumption data, the NOAEL still offers a 630 fold Margin of Safety.

Based on the results of safety studies and other evidence, CB108 Lactase 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 lowering the manufacturing cost and improving quality of final foods.

Approval of this application would provide manufacturers and/or consumers with benefits of facilitating dairy processing and GOS production, potentially 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:

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
Tel : 02 47875000 | Mobile:
Email:

Danisco Singapore Pte Ltd
21 Biopolis Road #06-21
Nucleos, South Tower
Singapore 138567
Tel: | Mobile:
Email:
(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 enzyme *Processing Aid*, subject of this application. The intended uses of the processing aid include dairy processing and production of galacto-oligosaccharides (GOS).

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.

CB108 Lactase, subject of this application, is intended for use in dairy processing and GOS production.

Currently no lactase from *B. subtilis* is permitted as a Processing Aid, however other enzymes including α-Acetolactate decarboxylase, α-Amylase, β-Amylase, Asparaginase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Maltogenic α-amylase, Metalloproteinase, Pullulanase, and Serine proteinase, from *B. subtilis,* including recombinant strains are listed in Schedule 18 as permitted enzymes. 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

CB108 Lactase is produced by submerged fermentation of *Bacillus subtilis* carrying the lactase gene from *Bifidobacterium bifidum* encoding the truncated wild-type lactase enzyme. The enzyme is characterized as a β-D-galactoside galactohydrolase (EC 3.2.1.23). 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 dairy processing and the production of galacto-oligosaccharide (GOS). In all the applications described, the enzyme will convert lactose into GOS and glucose.

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 lactase from *B. subtilis* 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 β-galactosidase from *Bifidobacterium bifidum* expressed in *B. subtilis* in the Food Standards Australia New Zealand 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 CB108 Lactase derived from *B. subtilis* containing the gene for lactase from *Bifidobacterium bifidum* in Australia/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 (CCI)

Certain (identified) technical and manufacturing information included in Appendix B1, B2, B3, B4, B5 Supplementary 1, Appendix D3, Appendix E 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 an Exclusive Commercial Capturable 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

CB108 Lactase produced by *B. subtlis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

CB108 Lactase derived from *B. subtilis* carrying the gene encoding the lactase from *Bifidobacterium bifidum* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in production of GOS and fresh dairy products by a panel of scientific experts in the USA. It has also been approved in Denmark and France, and in the process of being approved in Canada. Refer to Appendix D for details.

1.9. Statutory declaration

I,

of 7 Cleopatra Street, Blackheath NSW 2785, 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: _____

1.10. 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

Please refer to Appendix A for further details

2.1. Type of processing aid

The lactase (CB108 Lactase) enzyme is an enzyme produced by submerged fermentation of *B.subtilis*, carrying the lactase gene from *Bifidobacterium bifidum* encoding the truncated wild-type lactase enzyme.

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 β-D-galactoside galactohydrolase. Other names used are: β-galactosidase, Exo-(1->4)-beta-D-galactanase, beta-galactosidase, lactase (ambiguous), beta-lactosidase, maxilact, hydrolact, beta-D-lactosidase, S 2107, lactozym, trilactase, beta-D-galactanase, oryzatym, sumiklat, Milky Whey, CB108 Lactase.

- EC number: 3.2.1.23
- CAS number: 9031-11-2

Biological source: The lactase (CB108 Lactase) enzyme is an enzyme produced by submerged fermentation of *B. subtilis*, carrying the lactase gene from *Bifidobacterium bifidum* encoding the truncated wild-type lactase enzyme.

2.2.2 Marketing Name of the Processing Aid:

The marketing name of this product will depend on the application. Some example marketing names of CB108 Lactase are FoodPro® GOS and ZymStar™ GOS.

2.2.3 Molecular and Structural Formula:

CB108 Lactase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

In general, the technological need of the enzymatic conversion of galactosides like lactose with the help of galactosidase can be described as: hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides resulting in the constituent monosaccharide and glucose which results in deletion of lactose in low lactose/lactose-free products (dairy processing), formation of non-digestible galacto-oligosaccharides (GOS) fibre in prebiotic drinks and sports drinks (dairy processing) and production of galacto-oligosaccharides from lactose (GOS production). CB108 Lactase will be used as follows:

Dairy processing:

In the production of dairy products, the enzyme will convert lactose into GOS and glucose. CB108 Lactase will be able to generate GOS *in situ* in raw milk or whey even with low lactose

content (roughly 5% lactose content in milk), with as benefit to provide 1) a low lactose/lactose free dairy product with reduced total sugars and caloric content in the final dairy product and 2) enable dairy products to contain galacto-oligosaccharides prebiotic material. Example of final dairy products produced with enzymated milk or whey would be milk, milk drinks, yogurts, fermented milk drinks, cheese …etc.

GOS production:

CB108 Lactase will also be used in the production of purified GOS. Because of the configuration of their glycosidic bonds, GOS largely resist hydrolysis by salivary and intestinal digestive enzymes. GOS are classified as prebiotics, defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. The increased activity of these health-promoting bacteria results in a number of effects, both directly by the bacteria themselves or indirectly by the organic acids they produce via fermentation. Examples of effects are stimulation of immune functions, absorption of essential nutrients, and syntheses of certain vitamins. GOS is primarily used in infant formula to mimic the effect of the human milk oligosaccharides (HMOs) on babies. GOS are present in both human milk and bovine milk, but in low concentration especially in human milk.

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:

White to brown powder or brown liquid, depending on the application.

Substrate specificity:

The CB108 Lactase enzyme hydrolyzes the terminal non-reducing β-D-galactose residues in β-D-galactosides. It can use galactosides like lactose as a substrate. Lactose is hydrolyzed into galactose and glucose.

Activity:

The activity of CB108 Lactase is defined in BLU units/g. The principle of this assay method is that lactase hydrolyzes 2-nitrophenyl-β-D-galactopyranoside (ONPG) into 2-nitophenol (ONP) and galactose. The reaction is stopped after fifteen minutes with the sodium carbonate and the liberated ONP is measured in spectrophotometer.

Temperature optimum:

Approximately 55°C.

Thermal stability:

The enzyme is relatively stable for 10 minutes at 50°C, while it is inactivated after 10 minutes of incubation at 70°C.

pH optimum:

Approximately 6. and the enzyme is active in the range of pH 4-8.

pH stability:

The enzyme is active in the range of pH 4-8.

Interaction of the enzyme with different foods:

The CB108 Lactase 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:

CB108 Lactase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. The use levels of CB108 Lactase 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. However, as described in previous sections, use of CB108 Lactase will provide the nutritional benefit 1) a low lactose/lactose free dairy product with reduced total sugars and caloric content in the final dairy product and 2) enable dairy products to contain galacto-oligosaccharides prebiotic material, 3) including GOS for infant formula and adult food.

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 CB108 Lactase 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 CB108 Lactase 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
Production strain	Negative by test
Physical properties:	
Appearance	white to brown powder, or brown liquid, depending on the application

Standard for identity:

CB108 Lactase 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:

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, CB108 Lactase 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 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 of CB108 Lactase (also known as β-Galactosidase) shows a clear conserved Glyco_hydro_2' superfamily sequence domain, characteristic for beta-galactosidase, beta-mannosidase and beta-glucuronidase activities.

CB108 Lactase enzyme sequence is identical to one of the approved β-Galactosidase enzymes in Schedule 18 of the ANZ Food Standards Code, i.e. the *Bifidobacterium bifidum* one, approved as expressed in *Bacillus licheniformis*. CB108 Lactase is expressed in *Bacillus subtilis*, which is closely related to *B. licheniformis*. The identity among the FSANZ approved lactases range from 12% (*K. lactis* to *A. niger* LacE) to 51% (*A. niger* LacE to *A. oryzae*). It is good to realize that some microorganisms contain more than one version of β-Galactosidase. For example, the ANZ FSC approved β-Galactosidase from A. niger probably contains enzyme expressed from at least four different β-Galactosidase genes (LacA, LacB, LacC, and LacE). Even the β-Galactosidase

sequences within this one species show amino acid sequence variability, i.e. an alignment of these four *A. niger* β-Galactosidase amino acid sequences (LacA, LacB, LacC, and LacE) showed that these were 34-51% identical.

CB108 Lactase enzyme derived from *B. subtilis*, carrying the lactase gene from *B. bifidum* has been determined to be GRAS in the United States, and been introduced in Europe market since 2017. There have not been any adverse events reported since CB108 Lactase 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 lactase sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The majority of matches were beta-galactosidases, 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 lactase sequence was performed against the Uniprot animal toxin database. This yielded no matches. Therefore, the lactase sequence does not share homology with a known toxin or venom sequence. For details, please refer to Appendix B.

Safe Strain Lineage concept

DuPont IB has determined by scientific procedures that production organism *B. subtilis* is safe as a production organism as it pertains to the DuPont *B. subtilis* Safe Strain Lineage (see Appendix B2).

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.

Toxicological testing

Toxicology studies with CB108 Lactase produced by *B. subtilis* have not been conducted. Instead, the safety of CB108 Lactase from *B. subtilis* has been assessed using toxicology studies conducted on earlier strains of the DuPont *B. subtilis* Safe Strain Lineage. A review of toxicology

studies conducted with enzyme preparations produced by *B. subtilis* strains indicates that, regardless of the *B. subtilis* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ (Appendix B2 and Appendix B3). Due to the consistency of the findings from enzyme preparations derived from different *B. subtilis* strains, it is expected that any new enzyme preparation produced from *B. subtilis* strains would have a similar toxicological profile.

For the determination of the safety of CB108 Lactase, we use the results of toxicology studies conducted on a practically identical lactase from *B. bifidum* produced in *B. subtilis* strain (BIF917) (Appendix B2 and Appendix B3).

The toxicological Data Set

The BIF917 Lactase has been subjected to the following toxicology tests:

- Acute Oral LD_{50} (limit test) in rats
- 13-week oral (gavage) toxicity in CD rats
- *In vitro* Chromosomal Aberration Study with human peripheral blood lymphocytes
- Bacterial reverse mutation assay (Ames assay)

A review of all toxicology studies conducted with BIF917 Lactase enzyme preparation produced by *B. subtilis* indicates that it is not acute toxic, mutagenic, or clastogenic in genotoxicity assays and do not adversely affect any specific target organ. Collectively, the *B. subtilis* BIF917 toxicology data support the concept of Safe Strain Lineage for *B. subtilis* CB108. Daily administration of BIF917 by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg total protein/kg bw/day corresponding to 1416.4 mg TOS/kg bw/day. Therefore, toxicology data obtained from production strain BIF917 could be applied to CB108 and the extrapolation of toxicology information is in line with the Safe Strain Lineage concept of Pariza and Johnson (2001). 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 is a strain of *B. subtilis* which has been genetically modified by DuPont IB to overexpress a lactase gene from *B. bifidum*.

The species *Bacillus subtilis* is an accepted source of enzymes in the literature and pathogenic strains are not described in the Bergey Manual or in the ATCC and other catalogs. The species *Bacillus subtilis* 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 agent at work" and is also not present on the European Guideline 93/88/EEG, the list of pathogens from the Dutch Guidelines for Genetically Modified Organisms (COGEM), the German "*Berufsgenossenschaft der chemischen Industrie*", or the Belgian "VLAREM II". *B. subtilis* is accepted as a safe host for the construction of Risk Group I GMMs in several countries, like

Germany, The Netherlands, and accepted as a host of certified host-vector systems under the NIH Guidelines in the USA.

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

B. subtilis occurs ubiquitously in the environment (soil, water, plants and animals) and as a result can be also found in food. The bacterium has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991). For example, alpha-amylase enzyme preparations from *B. subtilis* have been used commercially since 1929, when they were used in the manufacture of chocolate syrup to reduce its viscosity (Reed, 1975). Recently, scientists with the US Food and Drug Administration (FDA) reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer *et al.* 2006). An extensive risk assessment of *B. subtilis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. subtilis* strains used for enzyme manufacture are neither pathogenic nor toxigenic to humans. It is, however, prudent to ascertain the safety of the production strain as certain food-borne illness related strains may produce surfactin, a membrane spanning lipopeptide and amylolysin, a heat-stable toxin regarded to be a virulence factor (Apetroaie-Constantin *et al.*, 2009; Logan, 2012).

Numerous oral toxicity, mutagenicity and carcinogenicity studies using enzyme products from *B. subtilis* 168-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

3.5. Genetic stability of the source organism

The parental strain of the production strain *B. subtilis* 168 and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont IB and its parental companies, and it has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using *B. subtilis* 168 and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable after more than 45 generations of fermentation for lactase production. Refer also section 3.6.

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

The production organism of the CB108 Lactase preparation, the subject of this submission, is *B. subtilis* strain CB108. It is derived by recombinant DNA methods from *B. subtilis* strain 168. The purpose of this genetic modification is to enhance lactase production levels. The donor organism is *B. bifidum*. Lactase expression cassette was integrated into the host genome. 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 plasmid copy number analysis, and PCR analysis. Broth samples were taken prior and after prolonged fermentation mimicking commercial fermentation conditions. Samples were then used for genomic DNA extraction and analysis. Plasmid copy number analysis shows no significant change in the plasmid copy number from the beginning of fermentation to end of fermentation. No change in flanking DNA sequence was observed between the genomic DNA samples extracted from shake flask culture before serial transfer culture and those extracted after 60 generations of serial shake flask culture. These results indicate that strain CB 108 maintains the plasmid DNA stably over at least 60 generations.

Full details of the genetic modifications and stability of the inserted genes 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), CB108 Lactase will be used in:

- 1 Dairy products (excluding butter and fats)
- Purified GOS produced using CB108 Lactase, would be used where permitted in the FSC in foods such as 2.9.1 Infant Formula Products, 2.9.3 Food for Infants, and 2.9.3-7 Formulated Supplementary Foods for Young Children

4.2. Levels of residues in food

The proposed application rate of CB108 Lactase in dairy processing is 151-601 mg TOS/kg milk or whey. The proposed application rate of CB108 Lactase in GOS production is 317-932 mg TOS/kg lactose.

DuPont IB expects CB108 Lactase to be inactivated or removed during the subsequent production and refining processes for all applications.

In dairy processing, CB108 Lactase typically performs its technological function in the raw milk or whey product during yogurt production process/fermentation process for production of GOS in-situ. CB108 Lactase is denatured by heat (95-141 °C, 3 sec-10 min.) in the pasteurisation step.

In GOS production, CB108 Lactase performs its technological function during the enzymatic conversion of lactose into GOS contributing to an improved and consistent product. CB108 Lactase is denatured by heat (80-95 °C, 5-15 min) in an inactivation 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 CB108 Lactase, 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: 2.25 mg TOS/kg body weight/day. The NOAEL has been determined for CB108 Lactase to be at 1000 mg total protein/kg bw/day (equivalent to 1416.4 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 630X 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:

- 5% of the tonnage of dairy products sold in Australia and New Zealand

In addition, GOS made using this processing aid are expected to be in 15% of the tonnage of infant formula sold in Australia and New Zealand.

4.4. Levels of residues in food in other countries

Applications and levels of use of the CB108 Lactase preparation in other countries is the same as presented in section 4.2.

5. References

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